Selectin-driven Leukocyte Recruitment and Chemokines Facilitate Metastasis

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Alexandra Hoos

aus

Deutschland

Promotionskomitee

Prof. Dr. Anne Müller (Vorsitz)

PD Dr. Lubor Borsig (Leitung)

Prof. Dr. Thierry Hennet

Prof. Dr. Reto Schwendener

Zürich, 2013

"Science is like a box of chocolates. You never know what you're gonna get."

(adapted from Forrest Gump)

Table of contents

SUMMARY

Tumor is a complex tissue composed of malignant and heterogeneous non-malignant cells. Tumor progression is determined by the dynamic interactions between these cells. Soluble factors secreted within the tumor microenvironment stimulate stromal cells and induce infiltration by inflammatory cells. High amounts of monocytes/macrophages, commonly found in various types of malignant cancer are associated with increased tumor cell extravasation and metastasis. A recent study reported that CCL2 recruits inflammatory monocytes to promote metastasis. Concerted action of chemokines and adhesion molecules (selectins) regulates the homing of leukocytes to target sites. However, the role of endogenous (non-tumor derived) selectin ligands in metastasis was not characterized.

To study the contribution of endogenous selectin ligands to metastasis, we used *Fuc-TVII*$^{-/-}$ mice which display defective selectin ligands. Reduced recruitment of monocytes to metastasizing tumor cells in *Fuc-TVII*$^{-/-}$ mice correlated with attenuated metastasis, suggesting the presence of endogenous selectin ligands on monocytes as a prerequisite for their capture at metastatic sites. Adoptive transfer of Fuc-TVII^{+} monocytes rescued metastasis, corroborating that monocyte recruitment is selectin ligand-dependent. Moreover, decreased CCL2 expression in Fuc-TVII deficient lung was linked to impaired monocyte recruitment, reduced tumor cell survival and attenuated metastasis. This study demonstrated that endogenous selectin ligands mediate the recruitment and activation of monocytes at the metastatic site and thereby facilitate metastasis.

To delineate the molecular mechanism governing the effect of CCL2 on metastasis, we used different mouse models. Together with Monika Wolf, we demonstrated that CCR2 expression not only on monocytes, but in particular on the endothelium, is crucial for metastasis. Tumor cell-derived CCL2 induced vascular permeability and enabled efficient tumor cell extravasation via CCR2 signaling. The absence of CCR2 on the endothelium abolished tumor cell extravasation even in the presence of CCR2^{+} monocytes, highlighting that activation of CCR2 on the endothelium is a prerequisite for tumor cell migration. Collectively, our study identified a novel mechanism for CCL2-dependent metastasis.

ZUSAMMENFASSUNG

Tumorgewebe ist ein komplexes Gebilde, das sich aus malignen und heterogenen nicht-malignen Zellen zusammensetzt. Dynamische Wechselwirkungen zwischen diesen Zellen beeinflussen das Tumorwachstum. Lösliche Faktoren, sezerniert von der Tumor-Mikroumgebung, stimulieren stromale Zellen und rufen eine Infiltration von Entzündungszellen hervor. Ein hoher Anteil an Monozyten/Makrophagen, wie er häufig bei verschiedensten Tumorarten vorkommt, korreliert mit einer erhöhten Extravasationsrate von Tumorzellen und folglich Metastasierung. Aktuelle Studien zeigen, dass die CCL2-abhängige Rekrutierung von Monozyten die Metastasierung fördert. Chemokine und Adhäsionsmoleküle (Selektine) regulieren auf abgestimmte Art und Weise die Einwanderung von Leukozyten in die Zielorgane. Die Rolle von endogenen (nicht von Tumorzellen stammenden) Selektin-Liganden während der Metastasierung wurde jedoch bisher nicht charakterisiert.

Um den Beitrag endogener Selektin-Liganden zur Metastasierung zu untersuchen, verwendeten wir *Fuc-TVII*$^{-/-}$ Mäuse, welche über nicht-funktionelle Selektin-Liganden verfügen. Verringerte Rekrutierung von Monozyten zu den metastasierenden Tumorzellen in *Fuc-TVII*$^{-/-}$ Mäusen korrelierte mit verringerter Metastasierung. Dies deutet darauf hin, dass die Anwesenheit von endogenen Selektin-Liganden auf der Oberfläche von Monozyten essentiell für das Anhaften an Metastasen-bildenden Stellen ist. Adoptiver Transfer von Fuc-TVII^{+} Monozyten verhalf zu erfolgreicher Metastasierung und bestätigte somit die Selektin-Ligand abhängige Rekrutierung von Monozyten. Weiterhin korrelierte verminderte CCL2-Expression in Fuc-TVII-negativen Lungen mit beeinträchtigter Monozyten-Rekrutierung, reduzierter Überlebensrate der Tumorzellen und somit verringerter Metastasierung. Diese Studie zeigt somit, dass endogene Selektin-Liganden die Rekrutierung und Aktivierung von Monozyten vermitteln und dadurch erheblich zur Beschleunigung der Metastasierung beitragen.

Um den molekularen Wirkmechanismus von CCL2 bei der Metastasierung zu verstehen, haben wir verschiedene Maus Modelle verwendet. Zusammen mit Monika Wolf haben wir demonstriert, dass CCR2-Expression nicht nur auf Monozyten sondern auch auf dem

Endothel für die Metastasierung entscheidend ist. Von Tumorzellen stammendes CCL2 initiiert die Durchlässigkeit der Blutgefäße und ermöglicht dadurch eine effiziente Extravasierung der Tumorzellen durch CCR2-vermittelte Signalübertragung. Die Abwesenheit von CCR2 auf dem Endothel verhinderte die Extravasierung von Tumorzellen sogar in der Anwesenheit von $CCR2^+$ Monozyten. Dies hebt die Aktivierung von CCR2 als Voraussetzung für die Extravasierung von Tumorzellen hervor. Zusammenfassend hat unsere Studie einen neuen Mechanismus der CCL2-abhängigen Metastasierung aufgedeckt.

LIST OF ABBREVIATIONS

BMDCs:	bone marrow-derived cells
bFGF:	basic fibroblast growth factor
CAFs:	cancer associated fibroblasts
CCL2:	CC chemokine 2
CCR2:	CC chemokine receptor 2
CD:	cluster of differentiation
CSF-1:	colony-stimulating factor-1
EGF:	epidermal growth factor
EC:	endothelial cell
ECM:	extracellular matrix
EMT:	epithelial-mesenchymal transition
ERK:	extracellular signal regulated kinase
ESL-1:	E-selectin ligand-1
FAK:	focal adhesion kinase
Fuc-T:	fucosyltransferase
GLYCAM1:	glycosylation dependent cell adhesion molecule 1
G-CSF:	granulocyte colony-stimulating factor
GM-CSF:	granulocyte-macrophage colony-stimulating factor
HEVs:	high endothelial venules
ICAM:	intercellular adhesion molecule
IFN:	interferon
IL:	interleukin
iNOS:	inducible nitric oxide synthase
i.v.:	intravenous
JAK2:	Janus kinase 2
LLC:	Lewis lung carcinoma
M1:	pro-inflammatory macrophages, M1 phenotype

M2:	pro-tumor macrophages, M2 phenotype
M-CSF:	macrophage colony-stimulating factor
MAPK:	mitogen-activated protein kinase
MC-38:	murine colon adenocarcinoma cells
MDSCs:	myeloid-derived suppressor cells
MHC:	major histocompatibility complex
MMP:	matrix metalloproteinase
N1:	pro-inflammatory neutrophils, N1 phenotype
N2:	pro-tumor neutrophils, N2 phenotype
NFκB:	nuclear factor kappa B
NK:	natural killer cells
PAF:	platelet-activating factor
p.i.:	post injection
PNAd:	peripheral lymph node addressin
PSGL-1:	P-selectin glycoprotein ligand-1
ROS:	reactive oxygen species
SDF1:	stromal cell-derived factor-1
sLe^x:	sialyl-$Lewis^x$
STAT:	signal transducers and activators of transcription
TAMs:	tumor-associated macrophages
TANs:	tumor-associated neutrophils
TNFα:	tumor necrosis factor alpha
TGF:	transforming growth factor
VEGF:	vascular endothelial growth factor
VEGFR:	vascular endothelial growth factor receptor
VCAM:	vascular cell adhesion molecule
uPAR:	urokinase plaminogen activator receptor
uPA:	urokinase plaminogen activator

INTRODUCTION

1. Metastasis

Metastasis is a multistep process that involves the spread of tumor cells from the primary tumor to distant organs. It comprises a complex set of events including increased invasiveness, intravasation into circulatory system, survival, adhesion, extravasation and colonization.[1-4] Earlier hypothesis considered metastatic dissemination as the final step of cancer progression. However, recent findings identified cancer spread as an early event suggesting that metastatic abilities of cells can be acquired outside the primary lesion and do not necessarily develop first with large tumors.[5] Thus, disseminated tumor cells might develop parallel to the primary tumor and display different genetically and phenotypically alterations due to the selection pressure at sites distant from the primary tumor.[6-8] Upon curative resection of the primary tumor metastasis become the major cause for cancer-related death and prompted substantial interest of researchers into cellular and molecular mechanisms governing metastasis.[9]

1.1. Metastatic cells in circulation

Despite continual release of millions of tumor cells into the circulation, only a few cells leaving the primary tumor can successfully establish distant metastases.[10,11] The majority of circulating tumor cells in the blood stream are rapidly eliminated.[9,12] After intravenous injection of radiolabelled B16 melanoma cells only 0.1 % of cells were viable 24 hours later and less than 0.01 % of cells were able to establish experimental lung metastasis.[10] Still, the question of how disseminating tumor cells survive and proliferate at a distant site is not yet answered.

When patients are diagnosed with cancer, they usually already have tumor cells in the circulation. During entry into the bloodstream, disseminating tumor cells interact with blood components including platelets and leukocytes thereby forming so called “tumor cell emboli”.[13-15] Clinical and experimental studies revealed that tissue factor expressed by tumor cells triggers thrombin formation through the interaction with coagulation factors VII and X

(FVII and FX).[16,17] Indeed, thrombotic events are frequently present in cancer patients and an increase of procoagulants in blood is a prognostic tool for a poor outcome.[18,19] Fibrinogen deficiency in mice reduced metastasis of B16 melanoma and Lewis lung carcinoma cells to the lung indicating the importance of fibrinogen in sustained adhesion and survival of tumor cells in the lung vasculature.[20] In addition, defective platelet activation mediated by the absence of platelet receptors (e.g GPVI, GPIβ, PAR4, Gαq) diminished metastasis in several mice models.[21-24] Likewise, inhibition of integrins on platelets, tumor, or endothelial cells, leads to reduced metastatic potential demonstrating a crucial role of integrins in tumor cell-mediated interactions with platelets, leukocytes and endothelial cells in metastasis.[25,26]

Tumor cell emboli protect tumor cells from immune surveillance and ensure a prolonged tumor cell survival within the bloodstream.[21,27] Co-incubation of tumor cells with platelets prevented tumor cell lysis by natural killer (NK) cells *in vitro* confirming the fact that NK cell-mediated elimination of target cells requires direct cell-cell contact.[27] Platelet-derived transforming growth factor beta (TGFβ) impaired NK cell reactivity *in vitro*, demonstrated by reduced granule mobilization, cytotoxicity and interferon γ (IFNγ) production.[28] Further, mice depleted with NK cells and inoculated with tumor cells showed no differences in metastasis between platelet depleted and control groups. Thus, platelets promote metastatic spread by forming a physical barrier and protecting tumor cells from NK-mediated clearance. In addition, platelets mediate tumor cell adhesion to the endothelium and assist in tumor cell extravasation.[20,29] A recent study revealed a novel role for platelets in cancer dissemination.[30] Platelets secreted TGFβ induced epithelial-mesenchymal-like transition (EMT) in tumor cells through direct tumor-cell-platelet contact. Consequently, tumor cells became more invasive and highly metastatic.[30] This study indicates that platelets do not only cooperate in tumor cell survival and transmigration but can also reprogram tumor cells through direct interactions.

Although the contribution of leukocytes to thrombus formation is well accepted, the molecular mechanisms underlying this process are only beginning to emerge (discussed later).[31-34] Activated platelets and locally stimulated endothelial cells secrete chemokines that recruit inflammatory leukocytes to the tumor site via chemokine signaling.[35] The initial association of

tumor cells with leukocytes, platelets and endothelial cells is mediated by selectins.[36,37] Selectin deficiency or inhibition of selectins attenuated metastasis in several mice models.[33,38,39] Selectin-dependent activation and aggregation of platelets imply a complex interplay between tumor cells, platelets, leukocytes and endothelial cells during circulation and extravasation.[31,34]

1.2. Tumor cell extravasation

Tumor cells seed to multiple organs, but only in a few organs can the metastatic tumor grow and develop metastases. The establishment of a permissive tumor microenvironment is required for the successful colonization.[40] The infiltration barriers as well as the composition of the microenvironment are tissue-dependent and might explain the metastatic tropism of some types of cancer.[4,12] Endothelial cells in the vasculature of different organs express different cell adhesion molecules.[41,42] Therefore, tumor cells with corresponding counterparts can utilize these specific receptor-ligand interactions to adhere to specific tissue.[3] For example, breast cancer cells could specifically adhere to the lung vasculature and increase lung metastasis by over-expressing metadherin.[43] In addition, different vascular structures in organs can influence the extravasation abilities of tumor cells. Organs like bone marrow and liver, with characteristic fenestrated capillaries, are readily invaded by tumor cells when compared to lung or brain. In the latter organs cells need to migrate through the tight layer of endothelial cells surrounded by a basement membrane.[3,44,45] Thus, tumor cells require specific mediators that facilitate the process of extravasation in these organs.[46,47] After extravasation, tumor cells can grow and establish a metastatic foci only if the microenvironment of the target site provides an adequate supply of growth factors and stimuli required for tumor cell proliferation.[12,48,49]

Since detection and treatment of micro/macro-metastasis remains challenging, agents that can prevent early steps of metastasis are in demand. Targeting tumor cell emboli formation and extravasation represent an interesting and feasible mechanism to intervene in the

metastatic process. Thus, understanding the mechanisms by which selectins potentiate metastasis is the prerequisite for a promising anti-cancer therapy.

2. Selectins

Selectins belong to a family of vascular cell adhesion molecules. The structure of selectins encompasses an intracellular domain that interacts with the cytoskeleton, a transmembrane domain and an extracellular domain which is organized in two or nine consensus repeats, an epidermal growth factor-like (EGF) domain and a C-type lectin domain.[50,51] There are three members of selectins: P-selectin, E-selectin and L-selectin. L-selectin is constitutively expressed on all myeloid cells, most lymphocytes and on a subset of natural killer cells.[52] P-selectin is stored in α-granules in unstimulated platelets and in Weibel-Palade bodies of resting endothelial cells. It is rapidly translocated to the cell surface upon cell stimulation.[53,54] In contrast, E-selectin is synthesized *de novo* in response to inflammatory stimuli and its expression is restricted to endothelial cells. In some tissues such as skin and functional microdomains in the bone marrow, E-selectin is expressed constitutively on endothelial cells.[55,56]

2.1. Physiological role of selectins

Physiological functions of selectins are well described for processes like inflammation, the immune response, homeostasis and wound healing. The best studied role of selectins includes leukocyte trafficking.[52,57] Mature lymphocytes permanently recirculate from the blood into secondary lymphoid organs to provide an effective immune response. L-selectin mediates the extravasation of lymphocytes from the blood system and their homing to high endothelial venules (HEVs) in lymph nodes and to Peyer`s patches (lymphoid tissue in the gut).[57] Loss of L-selectin prevents lymphocyte homing and results in diminished immune response.[58]

Selectins also regulate the recruitment of leukocytes to sites of acute inflammation or injury. In blood, circulating leukocytes are under conditions of high shear stress. Under normal

conditions, endothelial cells and circulating leukocytes are not adhesive.[59] In response to inflammatory stimuli, endothelial cells express P- and E-selectin that together with L-selectin on leukocytes mediate the initial capture and rolling of leukocytes and platelets along the endothelium.[59-61] Rolling involves rapid formation of bonds at the leading edge of the circulating cells with the endothelium and rapid bond dissociation at the trailing edge.[57] Synergistic effects of multiple low-affinity selectin ligands lead to high avidity which enables selectins to cause circulating leukocytes to begin rolling.[57,62,63] This initial contact of leukocytes with endothelium provides sufficient time for the cells to be exposed to activating stimuli present on the vessel wall or released locally and to transmit the activating signals through adjacent signaling molecule receptors (e.g. G-protein-coupled receptors).[64] Chemokines, cytokines or platelet-activating factor (PAF) induce leukocyte activation and trigger firm attachment to the endothelium which is mediated by binding of leukocyte integrins to intercellular adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAMs) present on endothelial cells. Changes in shape promote activated leukocytes to migrate through the endothelial cell junctions, a process that involves interaction with endothelial junction proteins.[59] Platelet-derived P-selectin assists the efficient interaction of leukocytes with the endothelium by stimulating the activation of these cells.[65] The multistep cascade of leukocyte recruitment is demonstrated in Figure 1.

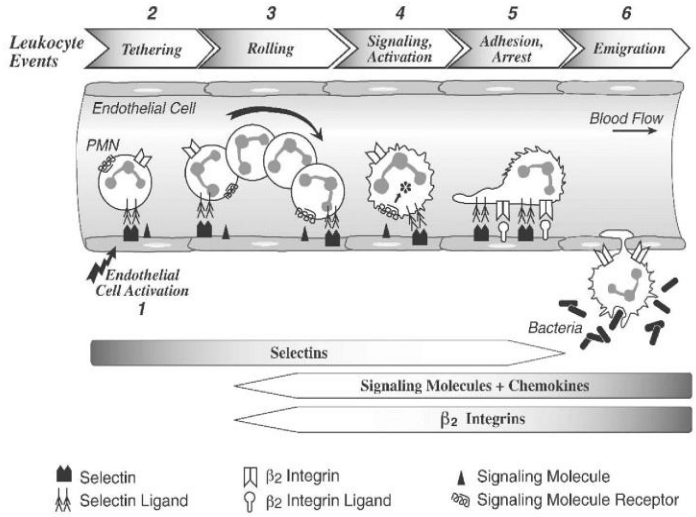


Figure 1. Leukocyte extravasation cascade.

Extravasation of leukocytes is initiated by the activation of endothelial cells, which expose E-and P-selectin and signaling molecules on the cell surface (1). The cascade is divided into several steps including capture (2), rolling (3), signaling activation (4) adhesion and arrest (5) followed by transmigration of leukocytes (6). Selectins mediate initial contact with the endothelium whereas firm adhesion of leukocytes is integrin dependent. After adhesion and crawling the cells finally transmigrate into the parenchyma. These events occur in a coordinated temporal fashion, ensuring spatial and temporal recruitment of leukocytes to the inflamed site. PMN: polymorphonuclear cells (McIntyre et al., 2003).

Interference with adhesive interactions at either the rolling, firm adhesion or transmigration steps decreased leukocyte migration and attenuates innate and adaptive immune responses during inflammation.[57,66] Interestingly, not all organs depend on a selectin-mediated adhesion cascade of leukocytes. For example, neutrophils were able to infiltrate lung and liver in P-selectin or E-selectin/P-selectin deficient mice. Whereas neutrophil recruitment to skin, skeletal muscle, heart and kidney, was dependent on selectin expression.[51,67,68]

Another prominent feature of selectins is their involvement in thrombosis. P-selectin expressed on activated platelets interacts with ligands on leukocytes and facilitates thrombi formation together with leukocytes.[69] Furthermore, P-selectin potentiates coagulation through the generation of leukocyte-derived microparticles.[70] Microparticles carry tissue factor that acts as key inducer of the extrinsic coagulation cascade.[71] P-selectin may also modulate

fibrin deposition and the resulting thrombus size.[70] The absence of P-selectin prolongs the bleeding time after injury confirming the role of P-selectin in thrombus formation.[72]

Various inflammatory stimuli like tumor necrosis factor-α (TNFα), interleukin-1β (IL1β) and histamine may stimulate the expression of L- and E-selectin as well as the degranulation of P-selectin.[52,73] Resting upon different regulatory mechanisms selectin expression and exposure on the cell surface is tightly controlled during homeostasis and ensures spatial and temporal recruitment of leukocytes during inflammation.

2.2. Selectin ligands

Selectins exert their functions through interactions with selectin ligands. Selectin ligands bind to the C-type lectin domain of selectins through specific carbohydrate determinants in a calcium-dependent manner. The minimal recognition motif for all selectins are the tetrasaccharides (Figure 2) sialyl-Lewis^x (sLe^x) and its isomer sialyl-Lewis^a (sLe^a).[74,75] sLe^x is sequentially synthesized by N-acetyl-glucosaminyltransferase, β1,4-galactosyltransferase, α2,3-sialyltransferase and α1,3-fucosyltransferase VII enzymes. N-acetyl-glucosaminyltransferase initiates synthesis of the sLe^x structure on poly-N-acetyllactosamines found on core O- and N-linked glycans displayed on proteins.[52,57,76]

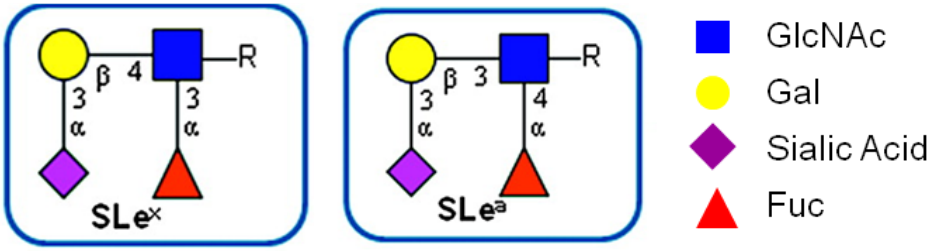


Figure 2. Structure of a sialyl-Lewis^x and sialyl-Lewis^a (adapted from Reis at al. 2010).

Carbohydrates with no sialic acid or fucose are poor ligands for selectins.[52,77] Additional post-translational modifications (e.g. glycosylation, sulfation) either on the sLe^x moiety or on the protein backbone potentiate selectin recognition of the ligand. Since all selectins share the same recognition motif, their selectivity depends on specific combinations of saccharides presented by the polypeptide backbone resulting in so called “clustered saccharide

patches".[77] Clustering of selectin ligands enhances the binding avidity of selectins and enables rapid contact formation. Highly glycosylated proteins facilitate the presentation of several selectin ligands in clusters. To date, the best characterized selectin ligand is P-selectin glycoprotein ligand-1 (PSGL-1).[78] PSGL-1 is expressed on leukocytes and is recognized by all three selectins[79-81], whereas the affinity for P- and L-selectin is increased by sulfation of tyrosines Tyr48 and Tyr51 near the N-terminus of the molecule.[82] Another selectin ligand, Glycosylation-dependent cell adhesion molecule-1 (GLYCAM1), requires sulfation of the carbohydrate chain for L-selectin recognition.[83]

Glycoproteins like GLYCAM1, endoglycan, podocalyxin and CD34 are highly expressed in HEVs. They belong to the so called peripheral lymph node addressin (PNAd) complex and regulate lymphocyte homing to the lymph nodes.[57]

Glycoproteins that mediate infiltration of leukocytes to sites of inflammation include PSGL-1, CD44, CD24 and E-selectin ligand-1 (ESL-1).[84-86] Leukocytes express an extensive repertoire of these glycoproteins on their surface. L-selectin on human neutrophils also carries carbohydrate determinants that are recognized by E-selectin. In contrast, mouse L-selectin does not bind to E-selectin indicating that selectin ligands in different species might have altered posttranslational modifications or are attached to different protein scaffolds.[87] It has been shown that PSGL-1 is responsible for initial leukocyte capture. ESL-1 converts the initial tethering, mediated by PSGL-1, into steady, slow rolling, whereas CD44 regulates the velocity of the slowly rolling leukocytes.[86] These data demonstrate how different selectin ligands cooperate together in a distinct and dynamic mode in order to promote leukocyte-endothelial interactions resulting in the extravasation of leukocytes to the inflammatory site.

The binding specificity of the same selectin ligand to its receptor might vary, depending on the cell-specific glycosylation pattern. CD44 expressed on hematopoietic progenitor cells was identified as a physiological L-and E-selectin ligand.[85,88,89] In contrast, T-cell-derived CD44 binds preferentially to hyaluronate and mediates T-cell extravasation to the site of inflammation.[90] The binding partner of endothelial-derived CD44 has not yet been identified.[50]

Alternatively, P- and L-selectin interact with sulfated ligands like heparin, heparin sulfate,

fucoidan and sulfated glycolipids.[77,91] Analysis of cell-specific expression of selectin ligands and their affinities for selectins could increase understanding of distinct functions.

Carcinoma cells display on their surfaces mucins with altered carbohydrate antigens.[92,93] Mucins are large proteins that bear many oligosaccharides packed closely together.[94] High expression of sLe^x and sLe^a is a major alteration on cancer mucins and correlates with tumor progression and metastasis.[95] Tumor associated sLe^x and sLe^a structures are major selectin ligands on cancer cells. Other tumor bearing selectin ligands like ESL-1, PSGL-1, death receptor 3, CD44 and CD24 have been reported. However, the biological relevance of these selectin ligands remains to be elucidated.

Most selectin counterparts have been identified using *in vitro* affinity purification techniques.[89,96] Binding affinity to a specific selectin type has been evaluated *in vitro* under static conditions rather than under fluid flow conditions which correspond more accurately to the natural processes found in the vasculature.[97] Only a few putative selectin ligands have been shown in animal models to mediate physiologically relevant interactions with selectins. Since only a few inhibitory antibodies are available, due to the poor immunogenicity of glycosylated epitopes, it remains challenging to prove the biological interaction of selectin ligands with their counterparts. Gene disruption is a commonly used strategy to ascertain the *in vivo* function of major selectin ligands.[98]

2.3. Selectin-selectin ligand-mediated signaling

Many studies have revealed different signaling pathways that are involved in selectin-mediated leukocyte extravasation. Selectin-selectin ligand interactions induce signaling in both the selectin-expressing and the ligand-expressing cells.[99-102] Selectin-mediated rolling of leukocytes stimulates a transient increase of Ca^{2+} in endothelial cells that is required for transit of cells across the endothelial cell barrier.[100] Focal adhesion kinase (FAK) has been activated in response to selectin binding of lymphocytes to the endothelial surface.[103] FAK promotes vascular permeability and up-regulation of endothelial adhesion molecules. Adhesion of myeloid cells to platelets via P-selectin-PSGL-1 interactions induces

translational amplification of urokinase plaminogen activator receptor (uPAR) in myeloid cells.[104] uPAR is an important protease receptor which regulates cell adhesion and migration during extravasation by coordinating extracellular matrix proteolysis and activating intracellular signaling pathways that support actin cytoskeleton reorganization.[105] P-selectin binding to human monocytes induces nuclear translocation of NF-kB that results in increased secretion of cytokines including TNF-alpha and CCL2 *in vitro*.[60] This observation may explain the contribution of selectins to local activation of cells during inflammation. Furthermore, E-selectin binding to leukocytes induces endothelial signal transduction and activation of Ras, Raf and extracellular signal-regulated kinases (ERK1/2) that in turn modulate the gene expression of adhesion molecules.[101] In contrast, L- and E-selectin-mediated leukocyte tethering and rolling on endothelial cells led to expression of integrins on leukocytes through the activation of p38, SYK and Src kinase pathways.[106-109] Engagement of different pathways suggests complex mechanisms for selectins contribution to cell-cell interactions. In addition, selectin-mediated interactions not only tether leukocytes, but also transmit outside-in signals that trigger morphological changes and alter gene expression.

2.4. Role of selectins in pathogenesis

Due to the involvement of selectins in various physiological situations (discussed above) aberrant selectin function is associated with many pathologies. Loss of individual or several selectins results in impaired leukocyte trafficking and causes increased susceptibility to opportunistic bacterial infections and toxic agents.[58,110] In contrast, each member of the selectin family (L-, P- and E-selectin) has been shown to facilitate acute inflammation and tissue damage in different disease models by virtue of enhanced leukocyte recruitment to the site of inflammation.[58,111,112] In patients with vascular disease and Crohn`s disease, soluble forms of P- and E-selectins were found in serum where they function as risk factors.[113] P-selectin neutralization abolished leukocyte infiltration and markedly decreased permeability of the small intestine in mice treated with endotoxin.[112] Inhibition of P and E-selectin protected ischemia/reperfusion-induced renal failure and showed reduced coagulation and

mortality in an experimental septic shock model.[111,114] Chronic inflammation is also associated with the ability of selectins to trigger thrombi formation. Studies of selectins in animal models revealed their involvement in the pathogenesis of atherosclerosis and arthritis. P-selectin expression on platelets facilitates platelet-monocyte aggregation and interaction of the aggregates within atherosclerotic lesions.[65] This selectin-mediated aggregation stimulates the local secretion of proinflammatory factors and strong infiltration of monocytes. Lack of P-selectin reduces platelet-induced monocyte recruitment on the endothelium and results in delayed onset of atherosclerosis.[65] The absence of L-and E-selectin also attenuated the progression of atherosclerotic lesions.[115-117] Sickle cell anemia is a disease in which red blood cells have an abnormal form accompanied by short life time, high stiffness and stickiness. P-selectin on endothelial cells accelerates sickle cell anemia by interacting with sickled erythrocytes.[118] Interestingly, vascular occlusion induced by sickle cell binding to firmly attached leukocytes on endothelium was absent in mice deficient both in P- and E-selectins.[119] Selectins are also involved in the coagulation abnormalities often observed in cancer patients.[120] Cancer patients are at high risk for thromboembolic events, a phenomenon that is known as Trousseau syndrome. Adenocarcinomas display and secrete high levels of mucins that can be recognized by selectins. Intravenous injection of purified carcinoma mucins triggered rapid platelet-rich microthrombi formation.[31] Deficiency either in P- or L-selectin reduced mucin-mediated platelet aggregation, indicating the importance of selectins in thrombotic events accompanying cancer progression.[31]

Collectively, these data indicate the role of selectins in regulatory processes of inflammation and cancer progression. Methods to block selectin-mediated interactions provide an efficient tool to prevent these events.

2.5. Fucosyltransferases

Fucosyltransferases are enzymes that catalyze the final step in the biosynthesis of selectin ligands.[84] To date, 11 fucosyltransferases (Fuc-Ts) have been identified. All 11 enzymes catalyze the transfer of fucose from GDP-fucose to various oligosaccharide acceptors. Depending on the linkage of fucose to the acceptor molecule, Fuc-Ts are divided into four groups. Fuc-TI and Fuc-TIl synthesize α1-2 fucose. Fuc-TIII, Fuc-TIV, Fuc-TV, Fuc-TVI, Fuc-TVII and Fuc-TIX are involved in the synthesis of α1-3/α1-4 fucose. Fuc-TVIII synthesizes α1-6 fucose. Fuc-TX and Fuc-TXI have been recently identified and their specific activity remains to be confirmed.[121] Two of these enzymes, Fuc-TIV and Fuc-TVII, are expressed in leukocytes and control the synthesis of selectin ligand sLe^x by transferring the fucose in α1,3-linkage to GlcNAc on the precursor moiety.[122] The importance of Fuc-TIV and Fuc-TVII in generation of selectin ligands has been identified in mice with targeted deletions in these genes. Mice deficient in the *Fuc-TVII* gene have a strong impairment in lymphocyte homing and in neutrophil infiltration into inflamed peritoneum.[123] The targeted loss of fucosylation leads to a marked reduction of selectin ligand activity on leukocytes and HEVs, demonstrating a central and essential role for Fuc-TVII in selectin ligand biosynthesis. Leukocytosis observed in *Fuc-TVII* deficient mice is mainly caused by increased neutrophil numbers (7-fold).[123] Elevated leukocyte numbers were also observed in P- and E-selectin double deficient mice.[110,124,125] Similar to *Fuc-TVII* deficient mice, patients with leukocyte adhesion deficiency type II (LADII), who carry mutations in the *Fuc-TVII* gene, showed impaired leukocyte interactions with the endothelium. As a result impaired wound healing and increased propensity to infection is commonly observed in these patients.[126,127] Taken together, this shows that the selectin-selectin ligand axis is required to maintain homeostasis of myeloid lineage leukocytes under normal physiological conditions.[84]

In contrast, the lack of *Fuc-TIV* has minimal effect on leukocyte trafficking. However, the retained selectin ligand activity in Fuc-TVII deficient neutrophils was abolished in mice deficient in both fucosyltransferases Fuc-TVII and Fuc-TIV. The residual lymphocyte homing activity to HEVs in *Fuc-TVII* deficient mice was strongly reduced in mice with the double

mutation.[128] The collaborative effect of Fuc-TIV and Fuc-TVII shows that both enzymes control the generation of selectin ligands, though Fuc-TVII has a dominating role over Fuc-TIV.[128] *In vitro* studies with synthetic polylactosamine precursors revealed distinct site-directed preferences between the Fuc-TVII and Fuc-TIV enzymes. Fuc-TIV preferentially fucosylates “inner“, whereas Fuc-TVII prefers “distal” lactoseamine units of precursors. These observations suggest that both enzymes contribute to selectin ligand synthesis in a site-specific manner.[129] Additionally, *in vivo* studies showed that Fuc-TIV preferentially generated sLe^x-like epitopes on glycolipids, which might explain why Fuc-TIV is less important in protein-based selectin ligand biosynthesis than Fuc-TVII.[130] It is likely that other ligands may still bind to selectins even if both fucosyltransferases Fuc-TVII and Fuc-TIV are missing.[131] However, the structure and the biological relevance of these ligands remain to be determined.

Interestingly, a recent study revealed that in human gastrointestinal tissues, as well as in colon cancer, Fuc-TVI is a key regulator of sLe^x biosynthesis, whereas the functions of Fuc-TIV and Fuc-TVII are negligible. This indicates that contributions from different fucosyltransferases to the biosynthesis of sLe^x are cell/tissue specific.[132]

2.6. Selectin-selectin ligand axis in tumor progression

During the hematogenous phase of metastasis, circulating cancer cells undergo a range of cell-cell interactions which determine tumor cell survival, adhesion and extravasation.

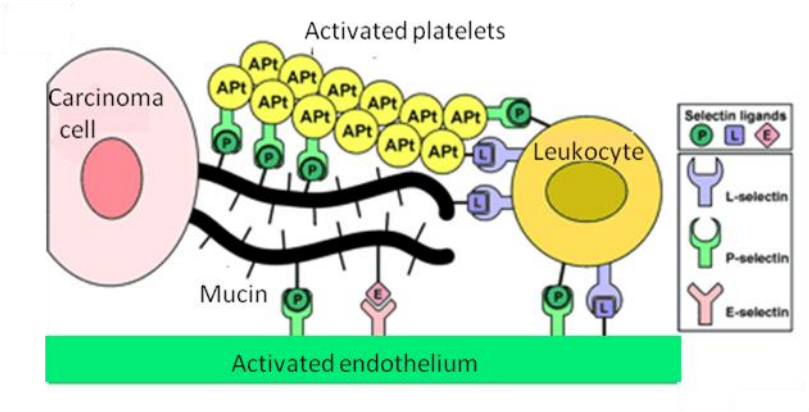


Figure 3. Potential interactions of a tumor cell with selectins.
Entering the blood stream, tumor cells interact with blood elements (cells and soluble factors) and the vascular endothelium. These interactions are determinants for metastasis. The schematic model depicts all possible interactions of carcinoma cell mucins with selectins present on blood constituents (platelets, leukocytes and endothelial cells) as well as selectin-mediated natural crosstalk between the host cells (adapted from Borsig 2004).

Cancer cells often express a unique repertoire of glycans.[133] For instance, malignant cells, especially adenocarcinomas, display aberrant forms and amounts of mucins as a consequence of deregulated enzymes that modify them.[93]

Clinical observations and experimental studies provide accumulating evidence for the role of a selectin-selectin ligand axis in cancer progression.[36,37] The majority of cancer cells, including melanomas, colon, gastric, breast and lung carcinomas display high levels of the selectin ligands sLe^x and sLe^a, and this is associated with progression and poor prognosis.[134] Numerous studies suggest that selectin-mediated processes might be exploited by the metastasizing tumor cells including the pro-coagulant activity, recruitment of leukocytes and stimulation of inflammatory pathways.[91] Mice deficient in one or more selectins show reduced metastasis and therefore confirm the involvement of all three selectin members in cancer.[32,33,135,136]

2.6.1. P-selectin in tumor progression

Cancer cell-mediated activation and binding of platelets is an important step in cancer progression.[13-15] The ability of tumor cells to form “tumor emboli” correlates with increased metastasis.[14,21,137] Attenuated metastasis and higher survival rate have been observed in mice with induced thrombocytopenia as well as in platelet-deficient mice.[21,22] How do platelets facilitate metastasis? Platelets aggregate around tumor cells and protect them from the immune system.[21,27] Animal models provided evidence that thrombi formation is primarily P-selectin dependent.[39,138] P-selectin deficiency reduces tumor cell-platelet interactions thereby causing a decreased tumor cell seeding to the lung.[39,138] Enzymatic removal of tumor cell mucins prior to intravenous injection resulted in decreased metastasis, indicating that P-selectin-mediated binding to mucins is essential for tumor cell-platelet aggregation.[138] The contribution of endothelial P-selectin to tumor cell-microvasculature interactions has been elucidated by the use of bone marrow reconstitution experiments in P-selectin-deficient mice.[135] Tumor cells have been shown to interact with postcapillary venules in a P-selectin-dependent manner, indicating that both, platelet and endothelial P-selectins, enhance metastasis.[135] Moreover, growth factors and cytokines released by activated platelets might supply tumor cells with critical factors to ensure their survival during circulation.[13-15] In addition, tumor microemboli interact with endothelial cells and thereby contribute to the arrest of tumor cells and their extravasation.[25,35,39]

Heparin, a highly sulfated glycosaminoglycan, prevented metastasis and improved patient survival.[139,140] Beyond its anticoagulant activity, heparin has been shown to attenuate metastasis in different tumor models due to its ability to block the binding of P-selectin to tumor cell glycans thereby preventing tumor cell emboli formation.[135,138] P- and L-selectin-mediated microthrombi formation was also associated with high thromboembolic events observed in patients with Trousseau syndrome that suffer from advanced cancer.[31] In addition, L-selectin-dependent interactions with mucins led to cathepsin G secretion in neutrophils.[34] Released cathepsin G in turn, activated platelets and triggered P-selectin expression.[34] Thus, tumor cells employ both, platelets and leukocytes in a selectin-

dependent manner and benefit from the reciprocal signaling of these cells, promoting thrombi formation and eventually metastasis.

2.6.2. L-selectin in tumor progression

High leukocyte infiltration in tumors has been associated with tumor progression and poor prognosis.[141-143] Inflammatory stimuli secreted by the tumor cell itself or by the surrounding microenvironment recruit leukocytes to primary tumor sites or metastatic lesions. Initial leukocyte response to tumors resembles, in general, the inflammatory response during infection or wound healing.[12,141,144] However, the impact of interactions between tumor and host cells within the bloodstream has been less explored. Recently, the interplay between tumor cells, platelets and leukocytes has been shown to stimulate an inflammatory microenvironment that activates the adjacent endothelial cells and triggers the recruitment of L-selectin-expressing leukocytes.[35] The absence of L-selectin resulted in attenuation of metastasis, implicating its role in this process.[33] Reduced recruitment of $CD11b^+$ leukocytes to tumor sites correlated with decreased tumor cell survival in the lung, confirming that L-selectin mediates leukocyte recruitment to metastatic sites.[33] The absence of L-selectin has further reduced metastasis in P-selectin deficient mice, implicating a synergistic action of P- and L-selectin in thrombi formation and metastasis, respectively.[32] L-selectin expressing leukocytes might also mediate the interaction of tumor cell emboli with L-selectin ligand expressing endothelial cells.[32] These findings indicate the ability of leukocytes to promote metastasis either by supporting tumor emboli formation or by tumor cell-endothelial interaction in an L-selectin dependent manner. Further evidence suggests that leukocytes assist tumor cells in breaching the endothelial barrier and might directly increase the migration of cancer cells.[145] The contribution of L-selectin-mediated interaction has been recently delineated (L.Borsig manuscript in preparation).

2.6.3. E-selectin in tumor progression

The local up-regulation of E-selectin in the vasculature in response to trapped tumor cell emboli has been observed in different mice models and with different tumor cell lines.[33,35,146] These observations imply that tumor cells are able to activate endothelial cells within the metastatic microenvironment and may exploit E-selectin interactions for successful colonization. Indeed, inhibition of E-selectin induced either by antisense oligonucleotides or neutralizing antibodies, reduces tumor cell adhesion and metastasis in several experimental metastasis models.[147-149] Interestingly, LS180 human colon carcinoma cells managed to metastasize and colonize the lung even in the absence of E-selectin, suggesting that different tumor cells apply different mechanisms for metastasis.[150] In addition, the metastatic microenvironment and the tissue-specific features of the target organ influence metastatic spread.[151] Over-expression of E-selectin in the liver of a transgenic mouse redirected tumor cell metastasis to this organ, clearly indicating the contribution of E-selectin to this process.[152] Endothelial E-selectin mediates tumor cell arrest by a direct interaction with E-selectin ligands displayed on tumor cells.[147,153] Expression of E-selectin ligands on tumor cells correlates directly with their enhanced adhesion to endothelial cells *in vitro.*[95,154] Accordingly, binding of E-selectin-ligand with a soluble form of E-selectin or with a peptide mimicking E-selectin ligand inhibits lung colonization by tumor cells.[38,155] However, tumor cells need first to activate the endothelial cells to trigger E-selectin interactions. In this context, tumor cells have been shown to co-opt inflammatory pathways followed by increased levels of cytokines within the metastatic microenvironment that ultimately promote E-selectin activation.[146] The importance of E-selectin in metastasis is also evident in regulation of cancer cell diapedesis through the endothelial cell layers.[156,157] The molecular mechanism of E-selectin-mediated tumor cell extravasation involves the activation of ERK and p38 mitogen-activated protein kinase (MAPK). In turn, activated kinases initiate a cascade of events leading to stress fibre formation and increased vascular permeability, thereby enabling the extravasation of adherent tumor cells.[157] Further, E-selectin up-regulation has been reported to initiate the homing of metastatic cancer cells to specific foci

in the lungs in a spontaneous breast metastasis model.[158] Cancer cell homing to lungs was abolished in E-selectin deficient mice, demonstrating the contribution of E-selectin to metastatic niche formation.[158] Thus, E-selectin not only promotes metastasis by guiding tumor cells and facilitating their adhesion to the vasculature, but it also transmits outside-in signals that support tumor cell extravasation.

2.6.4. Role of selectin ligands in tumor progression

Previous data showed strong evidence that P-, L- and E-selectin engagement with tumor cell selectin ligands facilitates metastasis. However, the role of endogenous selectin ligands expressed on host cells in metastasis formation has not yet been elucidated. It has been shown that neutrophils are able to roll on neutrophils already arrested on the endothelium.[159,160] The process of secondary capture is mediated exclusively by L-selectin and provides permanent leukocyte recruitment during inflammation. The phenomenon of secondary capture has been observed in cytokine-stimulated arterial vessels and on atherosclerotic lesions in the aorta.[161] L-selectin inhibition prevented secondary capture and decreased the flux of rolling leukocytes in arterial vessels.[161] These data clearly indicate the contribution of host-derived selectin ligands to the inflammatory response. However, the process of secondary capture in metastasis has not been studied. Furthermore, endogenous selectin ligands might be important in tumor emboli formation, based on selectin-mediated reciprocal signaling between platelets and leukocytes observed in a model of Trousseau syndrome.[34] The capacity of endogenous selectin ligands to promote metastasis has been reported recently.[33] Endogenous L-selectin ligands were up-regulated at the sites of intravascular tumor cell arrest in the lung of wild type mice. In contrast, no L-selectin ligands were detected around tumor cells in *Fuc-TVII*$^{-/-}$ mice. The lack of endogenous selectin ligands correlated with reduced lung metastasis in *Fuc-TVII*$^{-/-}$ mice.[33] Together, these data provide evidence that metastasis depends not only on direct selectin binding to tumor cells carrying selectin ligands, but also on the recognition of selectin ligands on host cells. These

interactions might activate intrinsic signaling cascades leading to amplification of the inflammatory response.[35]

3. Tumor microenvironment

Tumor is a complex tissue composed of malignant cells and multiple stromal cell types such as fibroblasts, epithelial cells, endothelial cells and infiltrating leukocytes. Initially, it was proposed that tumor cells alone drive carcinogenesis caused by neoplastic transformation. During the last decades, the supportive role of the tumor microenvironment became obvious. Current experimental and clinical evidence confirm that the interplay between tumor and non-malignant cells is crucial for tumor development at both, primary and metastatic sites.

3.1. Primary tumor microenvironment: composition and function

Tumor cells stimulate their microenvironment by direct cell-cell interactions and/or by secretion of soluble mediators, which allow them to educate the adjacent stromal cells. Stromal cells may originate from proliferated pre-existing stromal cells (e.g. epithelial cells, fibroblasts, endothelial cells), from *in situ* differentiated local stem cells or by recruitment of bone marrow-derived progenitor cells.[12,162] Upon stimulation, these cells secrete many factors that affect different aspects of tumor development, including tumor growth, angiogenesis, immunosuppression, invasiveness and metastasis.

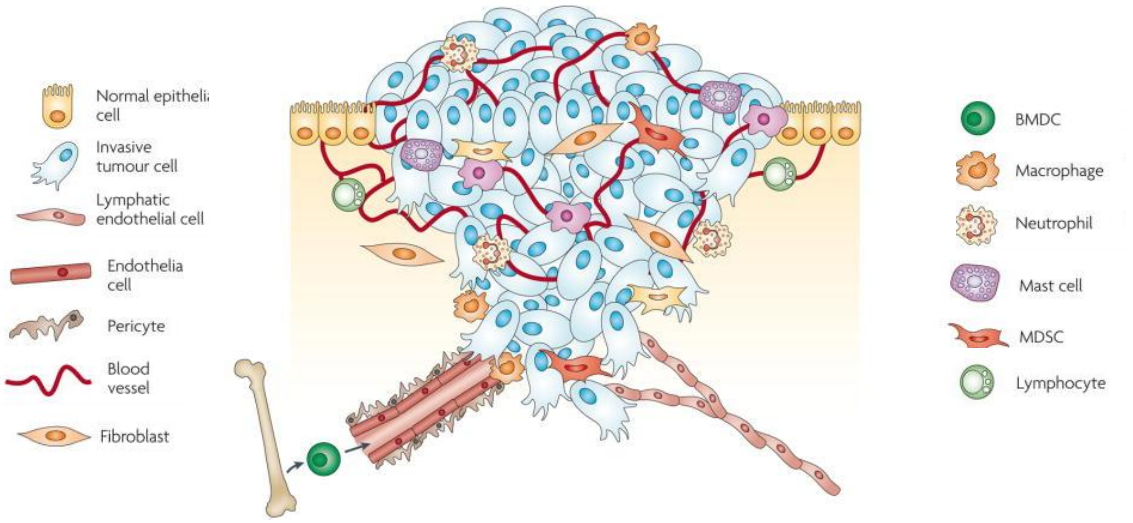


Figure 4. Composition of the primary tumor.

Primary tumor consists of cancer cells embedded in a complex network of stromal (left panel) and infiltrating (right panel) host cells. Dynamic interactions between these cells profoundly enhance tumor progression. The crosstalk supports tumor growth, angiogenesis, extracellular matrix degradation and metastasis through direct cell-cell contact and the production of a plethora of factors (e.g. cytokines, chemokines, growth factors and matrix-degrading enzymes, etc.). BMDC: bone marrow-derived cells. MDSC: myeloid-derived suppressor cells (Joyce et al. 2009).

3.1.1 Fibroblastic cells in tumor microenvironment

In solid tumors, cancer associated fibroblasts (CAFs) represent the major population of the stromal microenvironment.[163] There are suggestions that during tumor initiation CAF-derived proteases might support tumor growth by loosening the epithelial architecture and by degrading the growth suppression factors secreted by surrounding cells.[164] Indeed, CAF-derived proteases have been shown to release latent angiogenic factors like vascular endothelial growth factor (VEGF) or transforming growth factor β (TGFβ) sequestered in the ECM that in turn facilitated neovascularization and thereby promote tumor growth. CAFs might also secrete paracrine factors like VEGF or interleukin-8 that drive tumor angiogenesis.[165] Additionally, CAFs trigger angiogenesis either through direct recruitment of endothelial precursor cells via chemokine CXCL12, or by recruiting myeloid cells such as macrophages or neutrophils that produce proangiogenic factors.[166] Additional tumorigenic activities of CAFs are under investigation.

3.1.2 Endothelial cells in tumor microenvironment

Endothelial cells form tumor-associated vasculature which is required for nutrients and oxygen supply as well as disposal of metabolic waste.[167] VEGF, produced by tumor cells or CAFs within the tumor microenvironment, is a potent endothelial cell-specific mitogen and is crucial for tumor neovascularization. The angiogenic activity attributed to VEGF is, at least in part, based on its ability to induce apoptosis regulator protein Bcl-2 in endothelial cells.[168] Indeed, tumor-associated endothelial cells express high levels of Bcl-2.[169] Bcl-2 ensures enhanced endothelial cell survival and triggers secretion of chemokine CXCL1 and CXCL8 that in turn mediate signals promoting tumor angiogenesis and growth in head and neck squamous cell carcinomas. Further, the chemotactic gradient originating from endothelial cells is responsible for CXCR2-dependent tumor cell invasion.[170] Besides chemokines, endothelial cells secrete various factors including matrix metalloproteases (MMPs) and urokinase plaminogen activator (uPA) thereby supporting tumor growth and invasiveness. In addition, endothelial cells control the permeability of blood vessels and influence the egress of plasma proteins and cells that both directly and indirectly stimulate angiogenesis and other aspects of cancer.[171]

3.1.3 Functions of infiltrating leukocytes in tumor microenvironment

Bone marrow-derived cells make up a substantial proportion of cells within the tumor microenvironment. Different cell populations comprising T and B lymphocytes, mast cells, natural killer cells, dendritic cells, monocytes, granulocytes and partially differentiated myeloid progenitors have critical modulatory functions during both tumor development and metastasis.[12,172,173] Inflammatory cells within a tumor can incorporate both tumor-promoting as well as tumor killing subclasses. Since cancer progression positively correlates with inflammation, it is necessary to understand the complex interplay between tumor and inflammatory cells in order to obtain detailed insights into the nature of cancer.

In the following sections the importance of myeloid-derived suppressor cells, tumor-associated neutrophils and tumor-associated macrophages in the tumor microenvironment will be discussed in more detail.

3.1.3.1 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of cells that comprises myeloid progenitor cells, immature macrophages, immature granulocytes and immature dendritic cells.[174] MDSCs are excessively found in tumors where they accomplish different functions. The important function of MDSCs within the tumor microenvironment is their immunosuppressive activity.[174,175] MDSCs have been shown to suppress T cell functions by different mechanisms. For instance, high production of arginase 1 and inducible nitric oxide synthase (iNOS) enables MDSCs to inhibit major histocompatibility complex class II (MHC-II) expression and to induce apoptosis in T cells.[174,176,177] Further, MDSCs promote tumor angiogenesis.[174,178] They may trans-differentiate into endothelial-like cells and support blood vessel formation.[179] Elevated levels of signal transducer and activator of transcription 3 (STAT3) detected in MDSCs trigger the up-regulation of angiogenic genes like VEGF, basic fibroblast growth factor (bFGF) and MMPs.[180] Abrogation of STAT3 led to diminished MDSC-mediated angiogenesis. In addition, enhanced expression of MMP9 by MDSCs triggered the release of VEGF from the extracellular matrix stimulating the proliferation of endothelial cells.[181] Finally, in certain cellular contexts, MDSCs can differentiate into mature neutrophils or to mature macrophages and exert cell specific functions.[182]

3.1.3.2 Tumor-associated neutrophils

Tumor-associated neutrophils (TANs) have been detected in almost all tumors while their abundance varies depending on the type and size of a tumor. Several chemotactic factors such as CXCL1, CXCL2, CXCL5, granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) are secreted by both tumor and stromal cells and contribute to recruitment of neutrophils.[183,184] TANs in the tumor

microenvironment are activated and polarized either toward an N1 or N2 phenotype depending on the cytokine composition. An N1 phenotype is characterized by its antitumor activities such as high cytotoxicity and high expression levels of proinflammatory cytokines. The ability to mobilize and to activate $CD8^+$ T cells enhances the antitumor activities of N1 polarized neutrophils.[185] In contrast, N2 polarized neutrophils exert protumor activities due to a reduced cytotoxicity and increased secretion of immunosuppressive cytokines like IL6 and CCL17. Additionally, TANs in an N2 state release growth stimulating factors (e.g. VEGF, IL8, CCL2, CCL5) and matrix degrading proteases (e.g. MMP8, MMP9, elastase) that increase tumor cell survival and invasiveness.[185,186] Finally, N2 TANs have been shown to activate tumor angiogenesis by triggering the migration and proliferation of endothelial cells through the production of VEGF.[187] The depletion of neutrophils with anti-Gr1 antibody at the early stage of pancreatic islet tumorigenesis led to a reduced incidence of the “angiogenic switch”, demonstrating that neutrophils can switch on angiogenesis.[188] Interferon β (IFNβ) significantly shapes the ability of neutrophils to mediate angiogenesis. [189] The lack of IFNβ led to increased growth of melanoma and fibrosarcoma tumors which were associated with high levels of infiltrated neutrophils and enhanced blood vessel formation. Thus, IFNβ apparently primes the N1 phenotype, whereas the lack of IFNβ leads to generation of N2 TANs. In addition, the immunosuppressive cytokine TGFβ has an opposite effect on the plasticity of TANs.[185] The presence of TGFβ induces N2 polarization, while the absence of TGFβ leads to increased recruitment of neutrophils with higher cytotoxicity and production of proinflammatory cytokines typical for the N1 phenotype.[190] Indeed, in many tumors TANs have been shown to manifest N2 behavior that facilitates immunosuppression and tumor development. Collectively, these data provide conclusive evidence that tumor microenvironment educates TANs and shapes them with properties beyond their classical role.

3.1.3.3 Tumor-associated macrophages

Tumor-associated macrophages (TAMs) represent another dominant group of cells that populate primary tumors. Similar to neutrophils, circulating monocytes infiltrate tumors in response to high concentration of chemotactic factors such as CCL2, CCL5, macrophage colony-stimulating factor (M-CSF), VEGF and platelet-derived growth factor (PDGF).[144] Following tumor infiltration, monocytes differentiate into TAMs. Depending on the intratumoral composition of the inflammatory stimuli, TAMs may differentiate into M1 (classical) or M2 (alternative) phenotypes. Analogous to N1 and N2 neutrophils, M1 and M2 macrophages have opposing functions. IFNγ triggers M1 polarization of macrophages which secrete high levels of proinflammatory cytokines (TNFα, IL1, IL6, IL-12, IL23), MHC molecules and toxic intermediates such as nitric oxides and reactive oxygen species (ROS). Given these properties, M1 macrophages prime an antitumor immune response. Conversely, M2 macrophages shape a tumorigenic microenvironment by increased levels of anti-inflammatory cytokine IL10 and low levels of IL12. In addition, M2 macrophages create an attractive immunosuppressed environment for tumor growth by producing arginase, TGFβ and down-regulating the expression of MHC-II molecules. TAMs also represent an additional source of VEGF and other angiogenic factors like MMP9 and FGF which facilitate tumor angiogenesis.[179,191] M2 secreted MMPs, together with other proteolytic enzymes such as proteases and heparanases, degrade extracellular matrix and support tumor cell invasion and metastasis.[145,179,192-194] Moreover, stimulated macrophages release various chemokines that amplify and sustain pro-tumorigenic functions.[195]

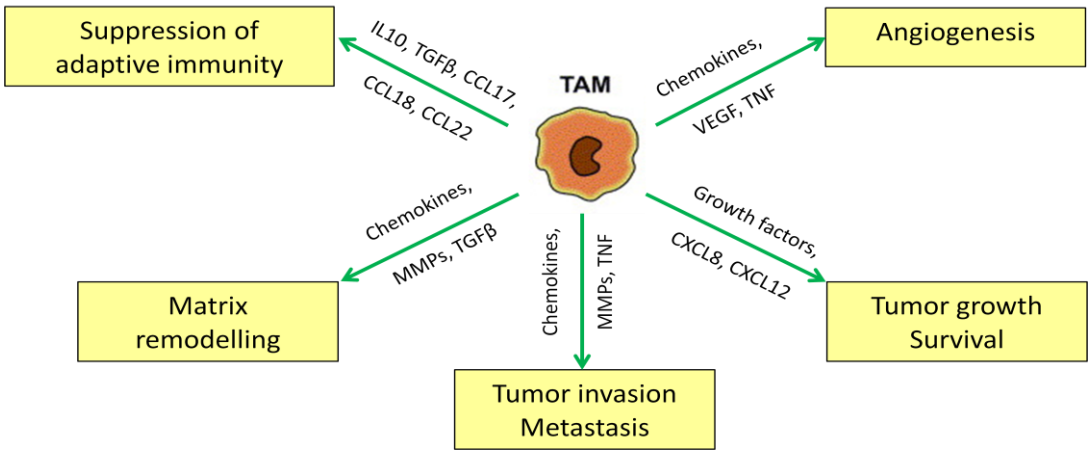


Figure 5: Protumorigenic functions of TAMs (Sica et al. 2006).

In the majority of investigated tumors, TAMs displayed an M2-like phenotype.[196] How the balance between tumor-suppressing and tumor-promoting activities of macrophages within the tumor is regulated is not completely understood. Tumor cells apply different mechanisms to educate TAMs toward a tumor-promoting state.[197,198] Anti-inflammatory molecules: IL10, IL4, IL13 and glucocorticoid hormones serve as important modulators of the M2 phenotype.[197] Intratumoral IL4 stimulates macrophages to produce high levels of cathepsins that are critical for promoting pancreatic tumor growth, angiogenesis, and invasion *in vivo*.[199] The function of TAMs in the tumor microenvironment is shaped both by tumor cells and other stromal cells (e.g. CAFs).[197,198] Recently, $CD4^+$ T cells were shown to cause M2 activation via IL-4 in a primary mammary carcinoma model.[200] Furthermore, the colony-stimulating factor (CSF-1)/EGF paracrine loop has been implicated as a possible mechanism of macrophage-mediated tumor cell invasion.[201,202] CSF-1 expressed by tumor cells promoted EGF secretion by macrophages. EGF, in turn, stimulated the formation of elongated protrusions in tumor cells leading to increased motility and invasion. Interference with the CSF-1/EGF paracrine loop resulted in reduced tumor cell invasiveness and showed antitumor effects *in vivo*.[203,204] Macrophage assistance during tumor cell invasion was confirmed by intravital imaging as direct interactions between macrophages and tumor cells were visualized in the invasive regions of the tumor.[205]

Characterization of intrinsic pathways responsible for TAM polarization are essential for understanding how these cells affect tumor progression.[142] Interestingly, the prominent transcription factors such as STAT3, STAT6, NF-κB and hypoxia inducible factor α (HIFα) have been identified as key orchestrators of M2 polarization.[206-209] Since TAMs preferentially accumulate in hypoxic areas, up-regulation of HIFα levels have been detected.[179] HIFα controls the production of angiogenic factors like VEGF, bFGF and chemokines such as CXCL8 and CXCL12, thereby promoting tumor progression and continuous recruitment of myeloid cells.[210,211] Consequently, ablation of HIFα diminished macrophage motility and cytotoxic activity under hypoxic conditions.[210] Taken together, these data demonstrate the impact of hypoxia on the recruitment and polarization of TAMs and highlight the contribution of the microenvironment to tumor progression.

3.2. Tumor microenvironment at the metastatic site

The dynamic interactions of tumor cells with cells in their environment occur not only in the primary tumor, but also during circulation in the blood stream and at distant sites of metastatic growth.[2,12] Following entry into circulation, disseminating tumor cells encounter a foreign microenvironment in the blood system and later at the distant site. Besides the appropriate genetic or epigenetic alterations that provide tumor cells with specific cues for survival, tumor cells apply different mechanisms to adapt and exploit cells in their vicinity. These mechanisms determine tumor cell survival and contribute to successful organ colonization.[2,9] For instance, tumor cells induce the up-regulation of vascular cell-adhesion molecules like selectins and integrins in order to identify and bind to vascular beds at secondary sites.[212,213] Tumor-mediated local activation of endothelial cells triggers the expression of inflammatory mediators which facilitate tumor cell extravasation and outgrowth.[146] Increased production of CCL5 by tumor-stimulated endothelial cells has been shown to recruit monocytes to metastatic sites.[35] Activated monocytes at metastatic sites secrete various factors like MMPs and caspases which facilitate the establishment of micrometastases.[12] Accumulating data implicate soluble factors produced by primary tumors

as mediators of molecular and physiological changes in distant organs that may direct the dissemination of malignant cells to the prepared metastatic sites = “metastatic niche”.[214-216]

3.2.1 Formation of the premetastatic niche

The existence of so called “premetastatic niches” has been described recently. Premetastatic niche is defined as the formation of a fertile environment at a distant site prior to the arrival of metastatic cancer cells.[217] VEGFR-1-expressing hematopoietic progenitor cells home to distinct sites and form receptive clusters that attract metastatic tumor cells.[214] These clusters not only have a growth-supporting function but also affect the metastatic tropism of the primary tumor. Primary tumors release high levels of VEGF into the circulation and recruit vascular endothelial growth factor receptor 1 (VEGFR-1) positive hematopoietic progenitor cells to the premetastatic niche. The VEGFR-1 function-blocking antibody inhibited formation of these clusters and prevented tumor metastasis.[214] Other studies revealed additional mechanisms that shape the premetastatic niche. Increased fibronectin expression by resident fibroblasts caused by tumor-specific growth factors allows very late antigen-4 (VLA-4)$^+$/VEGFR-1$^+$ hematopoietic progenitor cells to adhere. In response to fibronectin binding, activated VEGFR-1$^+$ cells enhance metalloproteinase expression and create permissive surroundings for incoming tumor cells.[214,218,219] Factors released from the primary tumor such as VEGF-A, TNFα and TGFβ induce enhanced production of inflammatory chemoattractants S100A8 and S100A9 that cause infiltration of leukocytes to the premetastatic lung.[215] Lysyl oxidase (LOX) secreted by hypoxic breast tumor has also been shown to accumulate in the premetastatic niche.[216] LOX is responsible for cross-linking collagen IV, thereby increasing the adhesion and accumulation of BMDCs at the site of future metastases.[216] In addition, stromal-derived factor-1 (SDF-1) is highly up-regulated in premetastatic clusters, and directs CXCR-4 expressing tumor cells and BMDCs to sites of metastasis.[220,221] Recently, tumor-derived exosomes have been shown to promote metastatic niche formation.[222] Exosomes are small membrane vesicles that mediate local and systemic cell communication by transferring their contents, including RNAs and proteins, to the cells they fuse with.[223]

Exosomes play an important role in mobilization of BMDCs and their reprogramming towards a pro-metastatic phenotype.[222] Of note, G-CSF functions as an alternative key initiator and regulator of metastatic niche formation.[224] Tumor-derived G-CSF stimulated the accumulation of $Ly6G^{+}Ly6C^{+}$ granulocytes in premetastatic tissue and enhanced the homing of breast tumor cells to lung.[224] These data indicate that primary tumors release several factors required for premetastatic niche formation, which probably consists of different cell populations of hematopoietic origin.

The significance of reciprocal interplay between host and tumor cells in tumor cell survival, proliferation and metastasis offers new approaches in anti-cancer therapy. Redirection of tumor-associated host cells toward tumor destruction represents one possible strategy. Although recent experimental studies provide positive results, the “re-programming” remains a difficult undertaking.[208,225] Since cellular composition and a variety of soluble factors determine the activation status of immune cells, further investigations are needed to understand the entire complexity of polarization mechanisms.

Another promising strategy aims to target extracellular mediators that act at the tumor-host communication interface.[226] Both tumor and host cells secrete a myriad of factors that promote tumor cell proliferation, migration, survival and extravasation. Clinical observations and animal studies demonstrate that dysregulation of chemokines and chemokine receptors enhances tumor development and progression, suggesting their role as prominent modulators of the tumor microenvironment.[141,195,227,228] Thus, chemokines and chemokine receptors might serve as potent target molecules in cancer therapy.

4. Chemokines

Chemotactic cytokines, also known as chemokines, are subdivided into 4 groups: CC, CXC, C and CX3C, according to the position of two highly conserved N-terminal cysteines.[229] Owing to their ability to regulate the migration of leukocytes, chemokines are involved in embryogenesis, organogenesis, tissue homeostasis and inflammation.[230] Alterations in chemokine expression have been associated with pathological conditions as shown in autoimmune disorders and infectious diseases like HIV or HCV.[231] In the last decade a great interest in chemokines has emerged as the novel role of chemokines in regulating various aspects of cancer has been demonstrated. The effects of chemokines in cancer progression are very complex and depend on their cell-specific origin and the appropriate microenvironment.

Many tumor cells aberrantly express chemokines. Chemokines can directly stimulate chemokine-receptor expressing tumor cells in an auto/paracrine manner (Figure 6). For example, overexpression of CXCL1 in normal melanoma cells accelerated cell proliferation *in vitro* and triggered tumor formation *in vivo*.[232] Along the same line, CXCL8 serves as a growth factor in pancreatic and head and neck carcinomas.[227,233] Beside the direct beneficial effects on tumor cells, angiogenic properties have been attributed to chemokines. CXCL5 and CXCL8 have been shown to act as pro-angiogenic factors, whereas CXCL9 und CXCL10 inhibited angiogenesis.

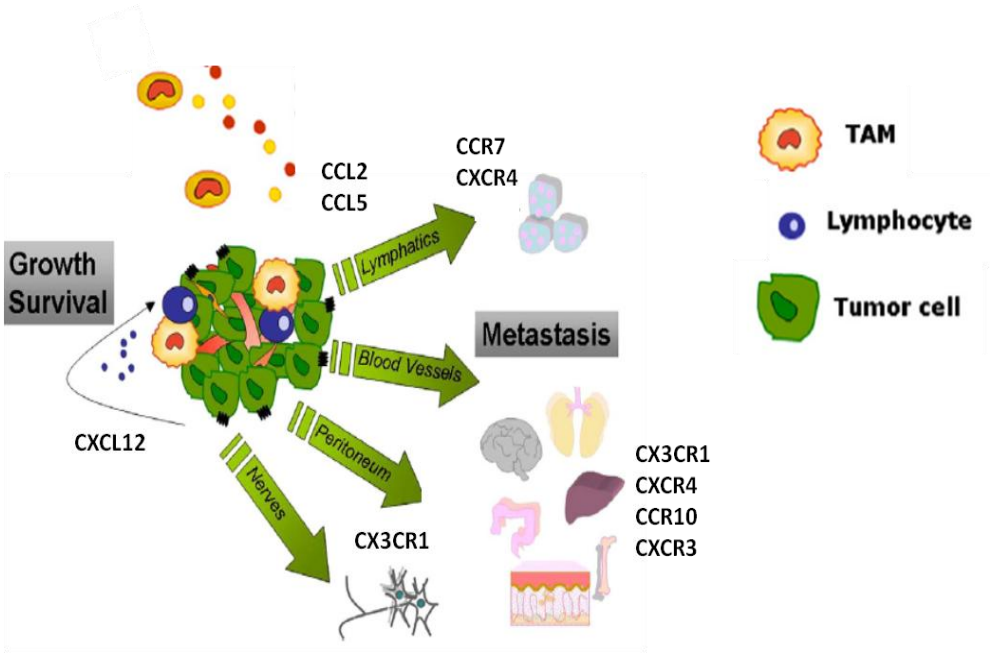


Figure 6: Direct and indirect effects of chemokines on tumor cells.
Tumor cell-derived chemokines (e.g. CXCL12) promote in a paracrine fashion tumor cell survival and proliferation. In addition, local chemokine gradient produced by tumor cells and stromal cells (CCL2, CCL5, CCL17, CCL22) triggers the recruitment of inflammatory monocytes and lymphocytes. This in turn facilitates tumor progression through the induction of angiogenesis, matrix remodeling and immune evasion. Chemokine receptors on tumor cells mediate their dissemination to distant organs (Allavena et al., 2011).

Chemokines not only influence local cells, but are also able to induce infiltration of leukocytes into the tumor. Up-regulation of CCL5 in a murine model of breast cancer, as well as in patients with breast and cervical cancer, was associated with enhanced infiltration of monocytes and increased metastasis, respectively.[234,235] In a different study, high levels of CCL20 in breast carcinomas led to strong infiltration of immature dendritic cells that correlated with poor clinical outcome.[236,237] Hence, the local production of various chemokines determines the amount and the composition of leukocytes in the tumor microenvironment.

Infiltrated leukocytes further amplify the inflammatory response by releasing pro-inflammatory cytokines, chemokines, growth factors and proteases, thereby promoting tumor development and metastasis. Among other properties that were summarized in section 3.1.3, TAMs, for instance, produce CCL18 that promotes lung and liver metastasis of breast cancer cells via integrin clustering, and enhanced adherence of metastasizing tumor cells to the

extracellular matrix.[238] Additionally, chemokines not only recruit inflammatory cells, but also may change the turnover and plasticity of these cells, thereby sustaining a tumor favoring microenvironment.[239]

Chemokines exert their pivotal functions through binding to corresponding G-protein-coupled receptors displayed on target cells. Proper signaling leads to intracellular calcium mobilization and chemotaxis.[228] Therefore, tight regulation of chemokine receptors on leukocytes is required to assure the trafficking and the retention of leukocytes in tumors. On the other hand, a particular repertoire of chemokine receptors on tumor cells may explain the tissue tropism typical for some tumor cell lines. For example, tissues with high SDF-1 expression such as bone, lungs and liver are favorable targets for metastasis of CXCR-4 expressing breast tumor cells.[220] The blockage of CXCR-4 signaling with neutralizing antibody abolished metastasis to these organs, indicating the chemokine-chemokine receptor axis as a critical factor for metastatic spread. In addition, inhibition of the chemokine receptor CXCR3 prevented lung metastasis in a murine model of metastatic breast cancer.[240] This indicates that various chemokine receptors may operate in parallel in order to promote cancer progression and metastasis.

Taken together, these data highlight the relevance of individual chemokines as well as chemokine-mediated local and systemic signaling in regulating tumor development and metastasis. Recently, CCL2 has been identified as a prominent regulator of metastasis.[241-243] CCL2 is produced by many cells like macrophages, fibroblasts, endothelial cells as well as tumor cells.[244] The mechanisms by which CCL2 promote metastasis are just beginning to be understood. In this context, the following section summarizes current findings on the impact of CCL2 in metastasis.

4.1 CCL2-CCR2 axis in tumor progression

High levels of CCL2 have been detected in tumors from colon, breast, liver, cervix and prostate cancer patients.[245-248] Overexpression of CCL2 in tumors correlates with metastasis and poor prognosis, indicating the importance of this chemokine in metastatic progression. Similarly, up-regulation of CCL2 in prostate cancer cells promoted tumor growth and bone metastasis due to increased recruitment of macrophages.[242] Another study showed that CCL2-mediated recruitment of monocytes facilitated breast cancer metastasis to the lung.[243] CCL2 blockage with neutralizing antibody was associated with reduced macrophage recruitment and reduced prostate tumor volume.[249] Attenuated metastasis and significantly prolonged survival was also observed in breast tumor-bearing mice after treatment with CCL2 neutralizing antibody.[243] Furthermore, nanoparticle-based silencing of CCR2 in monocytes was comparable with the effect caused by CCL2 neutralization and led to decreased macrophage recruitment in colorectal and lymphoma tumors.[250] Reduced lymphoma tumor volume was accompanied by low levels of VEGF and decreased vessel density.[250] Indeed, CCL2 has been shown to mediate angiogenesis of endothelial cells by enhancing the local concentration of VEGF released from recruited monocytes.[249] Further study demonstrated that CCL2 induced the formation of blood vessels in chick chorioallantoic membrane, indicating that CCL2 might directly stimulate vascularization through CCR2 signaling on the endothelial cells.[251] Recently, CCL2 activity has been linked to versican-mediated lung metastasis in bladder cancer.[252] High levels of versican potentiate metastasis in patients with bladder cancer and correlations between versican and CCL2 expression were detected in metastatic cohorts of human bladder tumors. Interestingly, metastasis suppressor molecule RhoGDI2 reduced versican expression and CCL2 secretion thereby diminishing macrophage recruitment to the lungs.[252] In addition, ablation of CCL2 or CCR2 attenuated lung colonization of highly metastatic versican-over-expressing bladder cancer cells. These data clearly indicate that the premetastatic effect of versican in bladder cancer is CCL2/CCR2 dependent. Further, macrophage depletion with clodronate liposomes

prevented versican-driven lung metastasis, suggesting that CCL2 function in versican-mediated lung metastasis is linked to macrophage infiltration.

Taken together, these data state the role of CCL2 as a facilitator of metastasis functioning through the recruitment of monocytes to tumor cells. However, the exact mechanism underlying CCL2 signaling during metastasis remains to be elucidated. The effect of CCL2 on other cells besides monocytes, awaits further characterization.

5. Scientific aims

5.1 Role of endogenous selectin ligands in metastasis

Metastasizing tumor cells exploit the immune system for successful colonization. Selectin ligands expressed by tumor cells have been shown to promote metastasis through selectin-mediated interactions with leukocytes.[32,136] In the current study, we aim to determine whether endogenous selectin ligands are also able to promote metastasis and to understand the molecular mechanism underlying this process. Insights into endogenous selectin ligand-mediated interactions may provide new strategies for preventing or controlling lung metastasis.

5.2 Impact of CCL2 on metastasis

High levels of CCL2 in tumors correlates with high metastatic potential in patients with prostate, lung and breast cancer. Recent studies associate the CCL2 metastasis promoting effect with the attraction of monocytes/macrophages to the tumor microenvironment. In the present study, we aim to analyze the role of CCL2-CCR2 axis in a murine metastatic model. Understanding CCL2-CCR2 signaling could help to develop new drugs and target metastasis at an early stage.

6. References

1. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002;2:563-572.
2. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. Nat Med. 2006;12:895-904.
3. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer. 2009;9:274-284.
4. Talmadge JE, Fidler IJ. AACR centennial series: the biology of cancer metastasis: historical perspective. Cancer Res. 2010;70:5649-5669.
5. Klein CA. Cancer. The metastasis cascade. Science. 2008;321:1785-1787.
6. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415:530-536.
7. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, Forni G, Eils R, Fehm T, Riethmuller G, Klein CA. Systemic spread is an early step in breast cancer. Cancer Cell. 2008;13:58-68.
8. Stoecklein NH, Hosch SB, Bezler M, Stern F, Hartmann CH, Vay C, Siegmund A, Scheunemann P, Schurr P, Knoefel WT, Verde PE, Reichelt U, Erbersdobler A, Grau R, Ullrich A, Izbicki JR, Klein CA. Direct genetic analysis of single disseminated cancer cells for prediction of outcome and therapy selection in esophageal cancer. Cancer Cell. 2008;13:441-453.
9. Mehlen P, Puisieux A. Metastasis: a question of life or death. Nat Rev Cancer. 2006;6:449-458.
10. Fidler IJ. Metastasis: guantitative analysis of distribution and fate of tumor embolilabeled with 125 I-5-iodo-2'-deoxyuridine. J Natl Cancer Inst. 1970;45:773-782.
11. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, Groom AC. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol. 1998;153:865-873.
12. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2009;9:239-252.
13. Nash GF, Turner LF, Scully MF, Kakkar AK. Platelets and cancer. Lancet Oncol. 2002;3:425-430.
14. Borsig L. The role of platelet activation in tumor metastasis. Expert Rev Anticancer Ther. 2008;8:1247-1255.
15. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. Nat Rev Cancer. 2011;11:123-134.
16. Mueller BM, Reisfeld RA, Edgington TS, Ruf W. Expression of tissue factor by melanoma cells promotes efficient hematogenous metastasis. Proc Natl Acad Sci U S A. 1992;89:11832-11836.
17. Ruf W, Mueller BM. Thrombin generation and the pathogenesis of cancer. Semin Thromb Hemost. 2006;32 Suppl 1:61-68.
18. Khorana AA, Fine RL. Pancreatic cancer and thromboembolic disease. Lancet Oncol. 2004;5:655-663.
19. Khorana AA, Connolly GC. Assessing risk of venous thromboembolism in the patient with cancer. J Clin Oncol. 2009;27:4839-4847.
20. Palumbo JS, Kombrinck KW, Drew AF, Grimes TS, Kiser JH, Degen JL, Bugge TH. Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. Blood. 2000;96:3302-3309.
21. Palumbo JS, Talmage KE, Massari JV, La Jeunesse CM, Flick MJ, Kombrinck KW, Jirouskova M, Degen JL. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. Blood. 2005;105:178-185.
22. Camerer E, Qazi AA, Duong DN, Cornelissen I, Advincula R, Coughlin SR. Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. Blood. 2004;104:397-401.

23. Jain S, Zuka M, Liu J, Russell S, Dent J, Guerrero JA, Forsyth J, Maruszak B, Gartner TK, Felding-Habermann B, Ware J. Platelet glycoprotein Ib alpha supports experimental lung metastasis. Proc Natl Acad Sci U S A. 2007;104:9024-9028.
24. Jain S, Russell S, Ware J. Platelet glycoprotein VI facilitates experimental lung metastasis in syngenic mouse models. J Thromb Haemost. 2009;7:1713-1717.
25. Trikha M, Zhou Z, Timar J, Raso E, Kennel M, Emmell E, Nakada MT. Multiple roles for platelet GPIIb/IIIa and alphavbeta3 integrins in tumor growth, angiogenesis, and metastasis. Cancer Res. 2002;62:2824-2833.
26. Bakewell SJ, Nestor P, Prasad S, Tomasson MH, Dowland N, Mehrotra M, Scarborough R, Kanter J, Abe K, Phillips D, Weilbaecher KN. Platelet and osteoclast beta3 integrins are critical for bone metastasis. Proc Natl Acad Sci U S A. 2003;100:14205-14210.
27. Nieswandt B, Hafner M, Echtenacher B, Mannel DN. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. Cancer Res. 1999;59:1295-1300.
28. Kopp HG, Placke T, Salih HR. Platelet-derived transforming growth factor-beta down-regulates NKG2D thereby inhibiting natural killer cell antitumor reactivity. Cancer Res. 2009;69:7775-7783.
29. Im JH, Fu W, Wang H, Bhatia SK, Hammer DA, Kowalska MA, Muschel RJ. Coagulation facilitates tumor cell spreading in the pulmonary vasculature during early metastatic colony formation. Cancer Res. 2004;64:8613-8619.
30. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell. 2011;20:576-590.
31. Wahrenbrock M, Borsig L, Le D, Varki N, Varki A. Selectin-mucin interactions as a probable molecular explanation for the association of Trousseau syndrome with mucinous adenocarcinomas. J Clin Invest. 2003;112:853-862.
32. Borsig L, Wong R, Hynes RO, Varki NM, Varki A. Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc Natl Acad Sci U S A. 2002;99:2193-2198.
33. Laubli H, Stevenson JL, Varki A, Varki NM, Borsig L. L-selectin facilitation of metastasis involves temporal induction of Fut7-dependent ligands at sites of tumor cell arrest. Cancer Res. 2006;66:1536-1542.
34. Shao B, Wahrenbrock MG, Yao L, David T, Coughlin SR, Xia L, Varki A, McEver RP. Carcinoma mucins trigger reciprocal activation of platelets and neutrophils in a murine model of Trousseau syndrome. Blood. 2011;118:4015-4023.
35. Laubli H, Spanaus KS, Borsig L. Selectin-mediated activation of endothelial cells induces expression of CCL5 and promotes metastasis through recruitment of monocytes. Blood. 2009;114:4583-4591.
36. Kim YJ, Borsig L, Han HL, Varki NM, Varki A. Distinct selectin ligands on colon carcinoma mucins can mediate pathological interactions among platelets, leukocytes, and endothelium. Am J Pathol. 1999;155:461-472.
37. Kannagi R. Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. Glycoconj J. 1997;14:577-584.
38. Mannori G, Santoro D, Carter L, Corless C, Nelson RM, Bevilacqua MP. Inhibition of colon carcinoma cell lung colony formation by a soluble form of E-selectin. Am J Pathol. 1997;151:233-243.
39. Kim YJ, Borsig L, Varki NM, Varki A. P-selectin deficiency attenuates tumor growth and metastasis. Proc Natl Acad Sci U S A. 1998;95:9325-9330.
40. Gupta GP, Minn AJ, Kang Y, Siegel PM, Serganova I, Cordon-Cardo C, Olshen AB, Gerald WL, Massague J. Identifying site-specific metastasis genes and functions. Cold Spring Harb Symp Quant Biol. 2005;70:149-158.
41. Nicolson GL. Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. Biochim Biophys Acta. 1988;948:175-224.
42. Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. Nature. 1996;380:364-366.

43. Brown DM, Ruoslahti E. Metadherin, a cell surface protein in breast tumors that mediates lung metastasis. Cancer Cell. 2004;5:365-374.
44. Paku S, Dome B, Toth R, Timar J. Organ-specificity of the extravasation process: an ultrastructural study. Clin Exp Metastasis. 2000;18:481-492.
45. Weil RJ, Palmieri DC, Bronder JL, Stark AM, Steeg PS. Breast cancer metastasis to the central nervous system. Am J Pathol. 2005;167:913-920.
46. Weis S, Cui J, Barnes L, Cheresh D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. J Cell Biol. 2004;167:223-229.
47. Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, Gomis RR, Manova-Todorova K, Massague J. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature. 2007;446:765-770.
48. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell. 1994;79:315-328.
49. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. Nat Rev Cancer. 2009;9:285-293.
50. Bevilacqua MP. Endothelial-leukocyte adhesion molecules. Annu Rev Immunol. 1993;11:767-804.
51. Ley K. The role of selectins in inflammation and disease. Trends Mol Med. 2003;9:263-268.
52. Kansas GS. Selectins and their ligands: current concepts and controversies. Blood. 1996;88:3259-3287.
53. Stenberg PE, McEver RP, Shuman MA, Jacques YV, Bainton DF. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. J Cell Biol. 1985;101:880-886.
54. Berman CL, Yeo EL, Wencel-Drake JD, Furie BC, Ginsberg MH, Furie B. A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterization and subcellular localization of platelet activation-dependent granule-external membrane protein. J Clin Invest. 1986;78:130-137.
55. Weninger W, Ulfman LH, Cheng G, Souchkova N, Quackenbush EJ, Lowe JB, von Andrian UH. Specialized contributions by alpha(1,3)-fucosyltransferase-IV and FucT-VII during leukocyte rolling in dermal microvessels. Immunity. 2000;12:665-676.
56. Sipkins DA, Wei X, Wu JW, Runnels JM, Cote D, Means TK, Luster AD, Scadden DT, Lin CP. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature. 2005;435:969-973.
57. Rosen SD. Ligands for L-selectin: homing, inflammation, and beyond. Annu Rev Immunol. 2004;22:129-156.
58. Tedder TF, Steeber DA, Pizcueta P. L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. J Exp Med. 1995;181:2259-2264.
59. McIntyre TM, Prescott SM, Weyrich AS, Zimmerman GA. Cell-cell interactions: leukocyte-endothelial interactions. Curr Opin Hematol. 2003;10:150-158.
60. Weyrich AS, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation. J Clin Invest. 1995;95:2297-2303.
61. Robinson SD, Frenette PS, Rayburn H, Cummiskey M, Ullman-Cullere M, Wagner DD, Hynes RO. Multiple, targeted deficiencies in selectins reveal a predominant role for P-selectin in leukocyte recruitment. Proc Natl Acad Sci U S A. 1999;96:11452-11457.
62. Vestweber D, Blanks JE. Mechanisms that regulate the function of the selectins and their ligands. Physiol Rev. 1999;79:181-213.
63. McEver RP, Zhu C. Rolling cell adhesion. Annu Rev Cell Dev Biol. 2009;26:363-396.
64. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007;7:678-689.

65. Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. Nat Med. 2003;9:61-67.
66. McEver RP, Moore KL, Cummings RD. Leukocyte trafficking mediated by selectin-carbohydrate interactions. J Biol Chem. 1995;270:11025-11028.
67. Wong J, Johnston B, Lee SS, Bullard DC, Smith CW, Beaudet AL, Kubes P. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. J Clin Invest. 1997;99:2782-2790.
68. Bullard DC, Qin L, Lorenzo I, Quinlin WM, Doyle NA, Bosse R, Vestweber D, Doerschuk CM, Beaudet AL. P-selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the peritoneum is completely absent but is normal into pulmonary alveoli. J Clin Invest. 1995;95:1782-1788.
69. Larsen PJ, Mikkelsen JD, Saermark T. Binding of a iodinated substance P analog to a NK-1 receptor on isolated cell membranes from rat anterior pituitary. Endocrinology. 1989;124:2548-2557.
70. Andre P, Hartwell D, Hrachovinova I, Saffaripour S, Wagner DD. Pro-coagulant state resulting from high levels of soluble P-selectin in blood. Proc Natl Acad Sci U S A. 2000;97:13835-13840.
71. Polgar J, Matuskova J, Wagner DD. The P-selectin, tissue factor, coagulation triad. J Thromb Haemost. 2005;3:1590-1596.
72. Subramaniam M, Frenette PS, Saffaripour S, Johnson RC, Hynes RO, Wagner DD. Defects in hemostasis in P-selectin-deficient mice. Blood. 1996;87:1238-1242.
73. Barthel SR, Gavino JD, Descheny L, Dimitroff CJ. Targeting selectins and selectin ligands in inflammation and cancer. Expert Opin Ther Targets. 2007;11:1473-1491.
74. Walz G, Aruffo A, Kolanus W, Bevilacqua M, Seed B. Recognition by ELAM-1 of the sialyl-Lex determinant on myeloid and tumor cells. Science. 1990;250:1132-1135.
75. Foxall C, Watson SR, Dowbenko D, Fennie C, Lasky LA, Kiso M, Hasegawa A, Asa D, Brandley BK. The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis(x) oligosaccharide. J Cell Biol. 1992;117:895-902.
76. Sperandio M, Gleissner CA, Ley K. Glycosylation in immune cell trafficking. Immunol Rev. 2009;230:97-113.
77. Varki A. Selectin ligands. Proc Natl Acad Sci U S A. 1994;91:7390-7397.
78. Wilkins PP, McEver RP, Cummings RD. Structures of the O-glycans on P-selectin glycoprotein ligand-1 from HL-60 cells. J Biol Chem. 1996;271:18732-18742.
79. McEver RP, Cummings RD. Role of PSGL-1 binding to selectins in leukocyte recruitment. J Clin Invest. 1997;100:S97-103.
80. Bernimoulin MP, Zeng XL, Abbal C, Giraud S, Martinez M, Michielin O, Schapira M, Spertini O. Molecular basis of leukocyte rolling on PSGL-1. Predominant role of core-2 O-glycans and of tyrosine sulfate residue 51. J Biol Chem. 2003;278:37-47.
81. Leppanen A, Yago T, Otto VI, McEver RP, Cummings RD. Model glycosulfopeptides from P-selectin glycoprotein ligand-1 require tyrosine sulfation and a core 2-branched O-glycan to bind to L-selectin. J Biol Chem. 2003;278:26391-26400.
82. Wilkins PP, Moore KL, McEver RP, Cummings RD. Tyrosine sulfation of P-selectin glycoprotein ligand-1 is required for high affinity binding to P-selectin. J Biol Chem. 1995;270:22677-22680.
83. Imai Y, Lasky LA, Rosen SD. Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. Nature. 1993;361:555-557.
84. Lowe JB. Selectin ligands, leukocyte trafficking, and fucosyltransferase genes. Kidney Int. 1997;51:1418-1426.
85. Katayama Y, Hidalgo A, Chang J, Peired A, Frenette PS. CD44 is a physiological E-selectin ligand on neutrophils. J Exp Med. 2005;201:1183-1189.
86. Hidalgo A, Peired AJ, Wild MK, Vestweber D, Frenette PS. Complete identification of E-selectin ligands on neutrophils reveals distinct functions of PSGL-1, ESL-1, and CD44. Immunity. 2007;26:477-489.

87. Zollner O, Lenter MC, Blanks JE, Borges E, Steegmaier M, Zerwes HG, Vestweber D. L-selectin from human, but not from mouse neutrophils binds directly to E-selectin. J Cell Biol. 1997;136:707-716.
88. Dimitroff CJ, Lee JY, Fuhlbrigge RC, Sackstein R. A distinct glycoform of CD44 is an L-selectin ligand on human hematopoietic cells. Proc Natl Acad Sci U S A. 2000;97:13841-13846.
89. Dimitroff CJ, Lee JY, Rafii S, Fuhlbrigge RC, Sackstein R. CD44 is a major E-selectin ligand on human hematopoietic progenitor cells. J Cell Biol. 2001;153:1277-1286.
90. DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science. 1997;278:672-675.
91. Laubli H, Borsig L. Selectins promote tumor metastasis. Semin Cancer Biol. 2010;20:169-177.
92. Fukuda M. Possible roles of tumor-associated carbohydrate antigens. Cancer Res. 1996;56:2237-2244.
93. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer. 2004;4:45-60.
94. Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD. An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell. 1992;69:927-938.
95. Takada A, Ohmori K, Yoneda T, Tsuyuoka K, Hasegawa A, Kiso M, Kannagi R. Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. Cancer Res. 1993;53:354-361.
96. Steegmaier M, Levinovitz A, Isenmann S, Borges E, Lenter M, Kocher HP, Kleuser B, Vestweber D. The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growth factor. Nature. 1995;373:615-620.
97. Lasky LA. Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science. 1992;258:964-969.
98. Yang J, Hirata T, Croce K, Merrill-Skoloff G, Tchernychev B, Williams E, Flaumenhaft R, Furie BC, Furie B. Targeted gene disruption demonstrates that P-selectin glycoprotein ligand 1 (PSGL-1) is required for P-selectin-mediated but not E-selectin-mediated neutrophil rolling and migration. J Exp Med. 1999;190:1769-1782.
99. Crockett-Torabi E. Selectins and mechanisms of signal transduction. J Leukoc Biol. 1998;63:1-14.
100. Lorenzon P, Vecile E, Nardon E, Ferrero E, Harlan JM, Tedesco F, Dobrina A. Endothelial cell E- and P-selectin and vascular cell adhesion molecule-1 function as signaling receptors. J Cell Biol. 1998;142:1381-1391.
101. Hu Y, Szente B, Kiely JM, Gimbrone MA, Jr. Molecular events in transmembrane signaling via E-selectin. SHP2 association, adaptor protein complex formation and ERK1/2 activation. J Biol Chem. 2001;276:48549-48553.
102. Urzainqui A, Serrador JM, Viedma F, Yanez-Mo M, Rodriguez A, Corbi AL, Alonso-Lebrero JL, Luque A, Deckert M, Vazquez J, Sanchez-Madrid F. ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. Immunity. 2002;17:401-412.
103. Haller H, Kunzendorf U, Sacherer K, Lindschau C, Walz G, Distler A, Luft FC. T cell adhesion to P-selectin induces tyrosine phosphorylation of pp125 focal adhesion kinase and other substrates. J Immunol. 1997;158:1061-1067.
104. Mahoney TS, Weyrich AS, Dixon DA, McIntyre T, Prescott SM, Zimmerman GA. Cell adhesion regulates gene expression at translational checkpoints in human myeloid leukocytes. Proc Natl Acad Sci U S A. 2001;98:10284-10289.
105. Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. Nat Rev Mol Cell Biol. 2009;11:23-36.
106. Yoshida M, Szente BE, Kiely JM, Rosenzweig A, Gimbrone MA, Jr. Phosphorylation of the cytoplasmic domain of E-selectin is regulated during leukocyte-endothelial adhesion. J Immunol. 1998;161:933-941.

107. Simon SI, Cherapanov V, Nadra I, Waddell TK, Seo SM, Wang Q, Doerschuk CM, Downey GP. Signaling functions of L-selectin in neutrophils: alterations in the cytoskeleton and colocalization with CD18. J Immunol. 1999;163:2891-2901.
108. Simon SI, Hu Y, Vestweber D, Smith CW. Neutrophil tethering on E-selectin activates beta 2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway. J Immunol. 2000;164:4348-4358.
109. Zarbock A, Abram CL, Hundt M, Altman A, Lowell CA, Ley K. PSGL-1 engagement by E-selectin signals through Src kinase Fgr and ITAM adapters DAP12 and FcR gamma to induce slow leukocyte rolling. J Exp Med. 2008;205:2339-2347.
110. Frenette PS, Mayadas TN, Rayburn H, Hynes RO, Wagner DD. Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. Cell. 1996;84:563-574.
111. Singbartl K, Green SA, Ley K. Blocking P-selectin protects from ischemia/reperfusion-induced acute renal failure. FASEB J. 2000;14:48-54.
112. Mangell P, Mihaescu A, Wang Y, Schramm R, Jeppsson B, Thorlacius H. Critical role of P-selectin-dependent leukocyte recruitment in endotoxin-induced intestinal barrier dysfunction in mice. Inflamm Res. 2007;56:189-194.
113. Demerath E, Towne B, Blangero J, Siervogel RM. The relationship of soluble ICAM-1, VCAM-1, P-selectin and E-selectin to cardiovascular disease risk factors in healthy men and women. Ann Hum Biol. 2001;28:664-678.
114. Norman KE, Cotter MJ, Stewart JB, Abbitt KB, Ali M, Wagner BE, Wallace WA, Forlow SB, Hellewell PG. Combined anticoagulant and antiselectin treatments prevent lethal intravascular coagulation. Blood. 2003;101:921-928.
115. Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL. P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. J Exp Med. 2000;191:189-194.
116. Galkina E, Kadl A, Sanders J, Varughese D, Sarembock IJ, Ley K. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. J Exp Med. 2006;203:1273-1282.
117. Gitlin JM, Homeister JW, Bulgrien J, Counselman J, Curtiss LK, Lowe JB, Boisvert WA. Disruption of tissue-specific fucosyltransferase VII, an enzyme necessary for selectin ligand synthesis, suppresses atherosclerosis in mice. Am J Pathol. 2009;174:343-350.
118. Matsui NM, Borsig L, Rosen SD, Yaghmai M, Varki A, Embury SH. P-selectin mediates the adhesion of sickle erythrocytes to the endothelium. Blood. 2001;98:1955-1962.
119. Turhan A, Weiss LA, Mohandas N, Coller BS, Frenette PS. Primary role for adherent leukocytes in sickle cell vascular occlusion: a new paradigm. Proc Natl Acad Sci U S A. 2002;99:3047-3051.
120. Rickles FR, Falanga A. Molecular basis for the relationship between thrombosis and cancer. Thromb Res. 2001;102:V215-224.
121. Miyoshi E, Moriwaki K, Nakagawa T. Biological function of fucosylation in cancer biology. J Biochem. 2008;143:725-729.
122. Breton C, Oriol R, Imberty A. Conserved structural features in eukaryotic and prokaryotic fucosyltransferases. Glycobiology. 1998;8:87-94.
123. Maly P, Thall A, Petryniak B, Rogers CE, Smith PL, Marks RM, Kelly RJ, Gersten KM, Cheng G, Saunders TL, Camper SA, Camphausen RT, Sullivan FX, Isogai Y, Hindsgaul O, von Andrian UH, Lowe JB. The alpha(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. Cell. 1996;86:643-653.
124. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. Cell. 1993;74:541-554.
125. Johnson RC, Mayadas TN, Frenette PS, Mebius RE, Subramaniam M, Lacasce A, Hynes RO, Wagner DD. Blood cell dynamics in P-selectin-deficient mice. Blood. 1995;86:1106-1114.
126. Bunting M, Harris ES, McIntyre TM, Prescott SM, Zimmerman GA. Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving beta 2 integrins and selectin ligands. Curr Opin Hematol. 2002;9:30-35.

127. Hidalgo A, Ma S, Peired AJ, Weiss LA, Cunningham-Rundles C, Frenette PS. Insights into leukocyte adhesion deficiency type 2 from a novel mutation in the GDP-fucose transporter gene. Blood. 2003;101:1705-1712.
128. Homeister JW, Thall AD, Petryniak B, Maly P, Rogers CE, Smith PL, Kelly RJ, Gersten KM, Askari SW, Cheng G, Smithson G, Marks RM, Misra AK, Hindsgaul O, von Andrian UH, Lowe JB. The alpha(1,3)fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. Immunity. 2001;15:115-126.
129. Niemela R, Natunen J, Majuri ML, Maaheimo H, Helin J, Lowe JB, Renkonen O, Renkonen R. Complementary acceptor and site specificities of Fuc-TIV and Fuc-TVII allow effective biosynthesis of sialyl-TriLex and related polylactosamines present on glycoprotein counterreceptors of selectins. J Biol Chem. 1998;273:4021-4026.
130. Huang MC, Laskowska A, Vestweber D, Wild MK. The alpha (1,3)-fucosyltransferase Fuc-TIV, but not Fuc-TVII, generates sialyl Lewis X-like epitopes preferentially on glycolipids. J Biol Chem. 2002;277:47786-47795.
131. Frenette PS, Moyna C, Hartwell DW, Lowe JB, Hynes RO, Wagner DD. Platelet-endothelial interactions in inflamed mesenteric venules. Blood. 1998;91:1318-1324.
132. Trinchera M, Malagolini N, Chiricolo M, Santini D, Minni F, Caretti A, Dall'olio F. The biosynthesis of the selectin-ligand sialyl Lewis x in colorectal cancer tissues is regulated by fucosyltransferase VI and can be inhibited by an RNA interference-based approach. Int J Biochem Cell Biol. 2010;43:130-139.
133. Fuster MM, Esko JD. The sweet and sour of cancer: glycans as novel therapeutic targets. Nat Rev Cancer. 2005;5:526-542.
134. Borsig L, Stevenson JL, Varki A. Heparin in Cancer: Role of Selectin Interactions. In: Khorana AA, Francis CW, eds. Cancer-Associated Thrombosis New York: Informa Healthcare; 2007:97-113.
135. Ludwig RJ, Boehme B, Podda M, Henschler R, Jager E, Tandi C, Boehncke WH, Zollner TM, Kaufmann R, Gille J. Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. Cancer Res. 2004;64:2743-2750.
136. Kohler S, Ullrich S, Richter U, Schumacher U. E-/P-selectins and colon carcinoma metastasis: first in vivo evidence for their crucial role in a clinically relevant model of spontaneous metastasis formation in the lung. Br J Cancer. 2009;102:602-609.
137. Honn KV, Tang DG, Crissman JD. Platelets and cancer metastasis: a causal relationship? Cancer Metastasis Rev. 1992;11:325-351.
138. Borsig L, Wong R, Feramisco J, Nadeau DR, Varki NM, Varki A. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci U S A. 2001;98:3352-3357.
139. Fielding LP, Hittinger R, Grace RH, Fry JS. Randomised controlled trial of adjuvant chemotherapy by portal-vein perfusion after curative resection for colorectal adenocarcinoma. Lancet. 1992;340:502-506.
140. Smorenburg SM, Hettiarachchi RJ, Vink R, Buller HR. The effects of unfractionated heparin on survival in patients with malignancy--a systematic review. J Thromb Haemost. 1999;82:1600-1604.
141. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860-867.
142. Talmadge JE, Donkor M, Scholar E. Inflammatory cell infiltration of tumors: Jekyll or Hyde. Cancer Metastasis Rev. 2007;26:373-400.
143. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454:436-444.
144. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. Cancer Metastasis Rev. 2006;25:315-322.
145. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141:39-51.
146. Khatib AM, Kontogiannea M, Fallavollita L, Jamison B, Meterissian S, Brodt P. Rapid induction of cytokine and E-selectin expression in the liver in response to metastatic tumor cells. Cancer Res. 1999;59:1356-1361.

147. Brodt P, Fallavollita L, Bresalier RS, Meterissian S, Norton CR, Wolitzky BA. Liver endothelial E-selectin mediates carcinoma cell adhesion and promotes liver metastasis. Int J Cancer. 1997;71:612-619.
148. Kobayashi K, Matsumoto S, Morishima T, Kawabe T, Okamoto T. Cimetidine inhibits cancer cell adhesion to endothelial cells and prevents metastasis by blocking E-selectin expression. Cancer Res. 2000;60:3978-3984.
149. Khatib AM, Fallavollita L, Wancewicz EV, Monia BP, Brodt P. Inhibition of hepatic endothelial E-selectin expression by C-raf antisense oligonucleotides blocks colorectal carcinoma liver metastasis. Cancer Res. 2002;62:5393-5398.
150. Laubli H, Borsig L. Selectins as mediators of lung metastasis. Cancer Microenviron. 2011;3:97-105.
151. Koike T, Kimura N, Miyazaki K, Yabuta T, Kumamoto K, Takenoshita S, Chen J, Kobayashi M, Hosokawa M, Taniguchi A, Kojima T, Ishida N, Kawakita M, Yamamoto H, Takematsu H, Suzuki A, Kozutsumi Y, Kannagi R. Hypoxia induces adhesion molecules on cancer cells: A missing link between Warburg effect and induction of selectin-ligand carbohydrates. Proc Natl Acad Sci U S A. 2004;101:8132-8137.
152. Biancone L, Araki M, Araki K, Vassalli P, Stamenkovic I. Redirection of tumor metastasis by expression of E-selectin in vivo. J Exp Med. 1996;183:581-587.
153. Laferriere J, Houle F, Huot J. Adhesion of HT-29 colon carcinoma cells to endothelial cells requires sequential events involving E-selectin and integrin beta4. Clin Exp Metastasis. 2004;21:257-264.
154. Tozeren A, Kleinman HK, Grant DS, Morales D, Mercurio AM, Byers SW. E-selectin-mediated dynamic interactions of breast- and colon-cancer cells with endothelial-cell monolayers. Int J Cancer. 1995;60:426-431.
155. Fukuda MN, Ohyama C, Lowitz K, Matsuo O, Pasqualini R, Ruoslahti E, Fukuda M. A peptide mimic of E-selectin ligand inhibits sialyl Lewis X-dependent lung colonization of tumor cells. Cancer Res. 2000;60:450-456.
156. Laferriere J, Houle F, Taher MM, Valerie K, Huot J. Transendothelial migration of colon carcinoma cells requires expression of E-selectin by endothelial cells and activation of stress-activated protein kinase-2 (SAPK2/p38) in the tumor cells. J Biol Chem. 2001;276:33762-33772.
157. Tremblay PL, Auger FA, Huot J. Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of p38 and ERK MAP kinases. Oncogene. 2006;25:6563-6573.
158. Hiratsuka S, Goel S, Kamoun WS, Maru Y, Fukumura D, Duda DG, Jain RK. Endothelial focal adhesion kinase mediates cancer cell homing to discrete regions of the lungs via E-selectin up-regulation. Proc Natl Acad Sci U S A. 2011;108:3725-3730.
159. Bargatze RF, Kurk S, Butcher EC, Jutila MA. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. J Exp Med. 1994;180:1785-1792.
160. Alon R, Fuhlbrigge RC, Finger EB, Springer TA. Interactions through L-selectin between leukocytes and adherent leukocytes nucleate rolling adhesions on selectins and VCAM-1 in shear flow. J Cell Biol. 1996;135:849-865.
161. Eriksson EE, Xie X, Werr J, Thoren P, Lindbom L. Importance of primary capture and L-selectin-dependent secondary capture in leukocyte accumulation in inflammation and atherosclerosis in vivo. J Exp Med. 2001;194:205-218.
162. Wels J, Kaplan RN, Rafii S, Lyden D. Migratory neighbors and distant invaders: tumor-associated niche cells. Genes Dev. 2008;22:559-574.
163. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006;6:392-401.
164. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell. 2012;21:309-322.
165. Rasanen K, Vaheri A. Activation of fibroblasts in cancer stroma. Exp Cell Res. 2010;316:2713-2722.
166. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas

promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121:335-348.
167. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000;407:249-257.
168. Nor JE, Christensen J, Mooney DJ, Polverini PJ. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol. 1999;154:375-384.
169. Kaneko T, Zhang Z, Mantellini MG, Karl E, Zeitlin B, Verhaegen M, Soengas MS, Lingen M, Strieter RM, Nunez G, Nor JE. Bcl-2 orchestrates a cross-talk between endothelial and tumor cells that promotes tumor growth. Cancer Res. 2007;67:9685-9693.
170. Warner KA, Miyazawa M, Cordeiro MM, Love WJ, Pinsky MS, Neiva KG, Spalding AC, Nor JE. Endothelial cells enhance tumor cell invasion through a crosstalk mediated by CXC chemokine signaling. Neoplasia. 2008;10:131-139.
171. Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. Nat Rev Cancer. 2010;10:138-146.
172. DeNardo DG, Johansson M, Coussens LM. Immune cells as mediators of solid tumor metastasis. Cancer Metastasis Rev. 2008;27:11-18.
173. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140:883-899.
174. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9:162-174.
175. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. J Immunol. 2001;166:5398-5406.
176. Rivoltini L, Carrabba M, Huber V, Castelli C, Novellino L, Dalerba P, Mortarini R, Arancia G, Anichini A, Fais S, Parmiani G. Immunity to cancer: attack and escape in T lymphocyte-tumor cell interaction. Immunol Rev. 2002;188:97-113.
177. Harari O, Liao JK. Inhibition of MHC II gene transcription by nitric oxide and antioxidants. Curr Pharm Des. 2004;10:893-898.
178. Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. Cancer Immunol Immunother. 2006;55:237-245.
179. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer. 2008;8:618-631.
180. Kujawski M, Kortylewski M, Lee H, Herrmann A, Kay H, Yu H. Stat3 mediates myeloid cell-dependent tumor angiogenesis in mice. J Clin Invest. 2008;118:3367-3377.
181. Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegue E, Song H, Vandenberg S, Johnson RS, Werb Z, Bergers G. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell. 2008;13:206-220.
182. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. J Immunol. 2005;175:4583-4592.
183. Kobayashi Y. The role of chemokines in neutrophil biology. Front Biosci. 2008;13:2400-2407.
184. Piccard H, Muschel RJ, Opdenakker G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. Crit Rev Oncol Hematol. 2011;82:296-309.
185. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell. 2009;16:183-194.
186. Fridlender ZG, Sun J, Mishalian I, Singhal S, Cheng G, Kapoor V, Horng W, Fridlender G, Bayuh R, Worthen GS, Albelda SM. Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. PLoS One. 2012;7:e31524.
187. Scapini P, Morini M, Tecchio C, Minghelli S, Di Carlo E, Tanghetti E, Albini A, Lowell C, Berton G, Noonan DM, Cassatella MA. CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. J Immunol. 2004;172:5034-5040.
188. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci U S A. 2006;103:12493-12498.

189. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. J Clin Invest. 2010;120:1151-1164.
190. Shen L, Smith JM, Shen Z, Eriksson M, Sentman C, Wira CR. Inhibition of human neutrophil degranulation by transforming growth factor-beta1. Clin Exp Immunol. 2007;149:155-161.
191. Lin EY, Pollard JW. Tumor-associated macrophages press the angiogenic switch in breast cancer. Cancer Res. 2007;67:5064-5066.
192. Murray GI, Duncan ME, O'Neil P, Melvin WT, Fothergill JE. Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. Nat Med. 1996;2:461-462.
193. Mohamed MM, Sloane BF. Cysteine cathepsins: multifunctional enzymes in cancer. Nat Rev Cancer. 2006;6:764-775.
194. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010;141:52-67.
195. Allavena P, Germano G, Marchesi F, Mantovani A. Chemokines in cancer related inflammation. Exp Cell Res. 2010;317:664-673.
196. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol. 2010;22:231-237.
197. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002;23:549-555.
198. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer. 2006;42:717-727.
199. Gocheva V, Wang HW, Gadea BB, Shree T, Hunter KE, Garfall AL, Berman T, Joyce JA. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. Genes Dev. 2010;24:241-255.
200. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell. 2009;16:91-102.
201. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res. 2004;64:7022-7029.
202. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, Stanley ER, Segall JE, Condeelis JS. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Res. 2005;65:5278-5283.
203. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med. 2001;193:727-740.
204. Aharinejad S, Paulus P, Sioud M, Hofmann M, Zins K, Schafer R, Stanley ER, Abraham D. Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice. Cancer Res. 2004;64:5378-5384.
205. Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, Segall JE, Pollard JW, Condeelis J. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res. 2007;67:2649-2656.
206. Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V, Firestein GS, Gerber HP, Ferrara N, Johnson RS. HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell. 2003;112:645-657.
207. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, Niu G, Kay H, Mule J, Kerr WG, Jove R, Pardoll D, Yu H. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med. 2005;11:1314-1321.
208. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. J Immunol. 2005;174:636-645.

209. Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG, Robinson SC, Balkwill FR. "Re-educating" tumor-associated macrophages by targeting NF-kappaB. J Exp Med. 2008;205:1261-1268.
210. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med. 2004;10:858-864.
211. Knowles H, Leek R, Harris AL. Macrophage infiltration and angiogenesis in human malignancy. Novartis Found Symp. 2004;256:189-200; discussion 200-184, 259-169.
212. Wang H, Fu W, Im JH, Zhou Z, Santoro SA, Iyer V, DiPersio CM, Yu QC, Quaranta V, Al-Mehdi A, Muschel RJ. Tumor cell alpha3beta1 integrin and vascular laminin-5 mediate pulmonary arrest and metastasis. J Cell Biol. 2004;164:935-941.
213. Auguste P, Fallavollita L, Wang N, Burnier J, Bikfalvi A, Brodt P. The host inflammatory response promotes liver metastasis by increasing tumor cell arrest and extravasation. Am J Pathol. 2007;170:1781-1792.
214. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature. 2005;438:820-827.
215. Hiratsuka S, Watanabe A, Aburatani H, Maru Y. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. Nat Cell Biol. 2006;8:1369-1375.
216. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, Le QT, Giaccia AJ. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell. 2009;15:35-44.
217. Peinado H, Marin F, Cubillo E, Stark HJ, Fusenig N, Nieto MA, Cano A. Snail and E47 repressors of E-cadherin induce distinct invasive and angiogenic properties in vivo. J Cell Sci. 2004;117:2827-2839.
218. Huhtala P, Humphries MJ, McCarthy JB, Tremble PM, Werb Z, Damsky CH. Cooperative signaling by alpha 5 beta 1 and alpha 4 beta 1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. J Cell Biol. 1995;129:867-879.
219. Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H, Shipley JM, Senior RM, Shibuya M. MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell. 2002;2:289-300.
220. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001;410:50-56.
221. Burger JA, Kipps TJ. Chemokine receptors and stromal cells in the homing and homeostasis of chronic lymphocytic leukemia B cells. Leuk Lymphoma. 2002;43:461-466.
222. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, Garcia-Santos G, Ghajar C, Nitadori-Hoshino A, Hoffman C, Badal K, Garcia BA, Callahan MK, Yuan J, Martins VR, Skog J, Kaplan RN, Brady MS, Wolchok JD, Chapman PB, Kang Y, Bromberg J, Lyden D. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med. 2012;18:883-891.
223. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9:654-659.
224. Kowanetz M, Wu X, Lee J, Tan M, Hagenbeek T, Qu X, Yu L, Ross J, Korsisaari N, Cao T, Bou-Reslan H, Kallop D, Weimer R, Ludlam MJ, Kaminker JS, Modrusan Z, van Bruggen N, Peale FV, Carano R, Meng YG, Ferrara N. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. Proc Natl Acad Sci U S A. 2010;107:21248-21255.
225. Guiducci C, Vicari AP, Sangaletti S, Trinchieri G, Colombo MP. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res. 2005;65:3437-3446.

226. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature. 2001;411:375-379.
227. Balkwill F. Chemokine biology in cancer. Semin Immunol. 2003;15:49-55.
228. O'Hayre M, Salanga CL, Handel TM, Allen SJ. Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. Biochem J. 2008;409:635-649.
229. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. Nat Immunol. 2001;2:123-128.
230. Karnoub AE, Weinberg RA. Chemokine networks and breast cancer metastasis. Breast Dis. 2006;26:75-85.
231. Zlotnik A, Yoshie O, Nomiyama H. The chemokine and chemokine receptor superfamilies and their molecular evolution. Genome Biol. 2006;7:243.
232. Dhawan P, Richmond A. Role of CXCL1 in tumorigenesis of melanoma. J Leukoc Biol. 2002;72:9-18.
233. Vicari AP, Caux C. Chemokines in cancer. Cytokine Growth Factor Rev. 2002;13:143-154.
234. Niwa Y, Akamatsu H, Niwa H, Sumi H, Ozaki Y, Abe A. Correlation of tissue and plasma RANTES levels with disease course in patients with breast or cervical cancer. Clin Cancer Res. 2001;7:285-289.
235. Stormes KA, Lemken CA, Lepre JV, Marinucci MN, Kurt RA. Inhibition of metastasis by inhibition of tumor-derived CCL5. Breast Cancer Res Treat. 2005;89:209-212.
236. Bell D, Chomarat P, Broyles D, Netto G, Harb GM, Lebecque S, Valladeau J, Davoust J, Palucka KA, Banchereau J. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. J Exp Med. 1999;190:1417-1426.
237. Treilleux I, Blay JY, Bendriss-Vermare N, Ray-Coquard I, Bachelot T, Guastalla JP, Bremond A, Goddard S, Pin JJ, Barthelemy-Dubois C, Lebecque S. Dendritic cell infiltration and prognosis of early stage breast cancer. Clin Cancer Res. 2004;10:7466-7474.
238. Chen J, Yao Y, Gong C, Yu F, Su S, Liu B, Deng H, Wang F, Lin L, Yao H, Su F, Anderson KS, Liu Q, Ewen ME, Yao X, Song E. CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. Cancer Cell. 2011;19:541-555.
239. Sawanobori Y, Ueha S, Kurachi M, Shimaoka T, Talmadge JE, Abe J, Shono Y, Kitabatake M, Kakimi K, Mukaida N, Matsushima K. Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. Blood. 2008;111:5457-5466.
240. Walser TC, Rifat S, Ma X, Kundu N, Ward C, Goloubeva O, Johnson MG, Medina JC, Collins TL, Fulton AM. Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. Cancer Res. 2006;66:7701-7707.
241. Loberg RD, Day LL, Harwood J, Ying C, St John LN, Giles R, Neeley CK, Pienta KJ. CCL2 is a potent regulator of prostate cancer cell migration and proliferation. Neoplasia. 2006;8:578-586.
242. Mizutani K, Sud S, McGregor NA, Martinovski G, Rice BT, Craig MJ, Varsos ZS, Roca H, Pienta KJ. The chemokine CCL2 increases prostate tumor growth and bone metastasis through macrophage and osteoclast recruitment. Neoplasia. 2009;11:1235-1242.
243. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA, Pollard JW. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature. 2011;475:222-225.
244. Yadav A, Saini V, Arora S. MCP-1: chemoattractant with a role beyond immunity: a review. Clin Chim Acta. 2010;411:1570-1579.
245. Zijlmans HJ, Fleuren GJ, Baelde HJ, Eilers PH, Kenter GG, Gorter A. The absence of CCL2 expression in cervical carcinoma is associated with increased survival and loss of heterozygosity at 17q11.2. J Pathol. 2006;208:507-517.
246. Yoshidome H, Kohno H, Shida T, Kimura F, Shimizu H, Ohtsuka M, Nakatani Y, Miyazaki M. Significance of monocyte chemoattractant protein-1 in angiogenesis and survival in colorectal liver metastases. Int J Oncol. 2009;34:923-930.
247. Zhang J, Patel L, Pienta KJ. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. Cytokine Growth Factor Rev. 2009;21:41-48.

248. Soria G, Ofri-Shahak M, Haas I, Yaal-Hahoshen N, Leider-Trejo L, Leibovich-Rivkin T, Weitzenfeld P, Meshel T, Shabtai E, Gutman M, Ben-Baruch A. Inflammatory mediators in breast cancer: coordinated expression of TNFalpha & IL-1beta with CCL2 & CCL5 and effects on epithelial-to-mesenchymal transition. BMC Cancer. 2011;11:130.
249. Loberg RD, Ying C, Craig M, Day LL, Sargent E, Neeley C, Wojno K, Snyder LA, Yan L, Pienta KJ. Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. Cancer Res. 2007;67:9417-9424.
250. Leuschner F, Dutta P, Gorbatov R, Novobrantseva TI, Donahoe JS, Courties G, Lee KM, Kim JI, Markmann JF, Marinelli B, Panizzi P, Lee WW, Iwamoto Y, Milstein S, Epstein-Barash H, Cantley W, Wong J, Cortez-Retamozo V, Newton A, Love K, Libby P, Pittet MJ, Swirski FK, Koteliansky V, Langer R, Weissleder R, Anderson DG, Nahrendorf M. Therapeutic siRNA silencing in inflammatory monocytes in mice. Nat Biotechnol. 2011;29:1005-1010.
251. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood. 2000;96:34-40.
252. Said N, Sanchez-Carbayo M, Smith SC, Theodorescu D. RhoGDI2 suppresses lung metastasis in mice by reducing tumor versican expression and macrophage infiltration. J Clin Invest. 2012;122:1503-1518.

RESULTS

Manuscript 1

Endothelial CCR2 Signaling Induced by Colon Carcinoma Cells Enables Extravasation via the JAK2-Stat5 and p38MAPK Pathway

Article published in Journal of Cancer Cell, 2012

Authors: Alexandra Hoos*, Monika Julia Wolf*, Judith Bauer, Steffen Boettcher, Markus Knust, Achim Weber, Nicole Simonavicius, Christoph Schneider, Matthias Lang, Michael Stürzl, Roland S. Croner, Andreas Konrad, Markus G. Manz, Holger Moch, Adriano Aguzzi, Geert van Loo, Manolis Pasparakis, Marco Prinz, Lubor Borsig*, and Mathias Heikenwalder*

* These authors contributed equally to this work

Contributions: I planned, conducted and analyzed the data summarized in figure 1A&G, 2A-B,D-E, 3C, 4C-D, 5A, F-G, 6A, 7A-E, G, 8B-C and parts of figure 3A and 6B. M.J.W. planned, conducted and analyzed the data summarized in figure 1C-D, F, 2C, 3A-B, 4A-B, 5D-E, 7F, 8D-E, helped with mice work and wrote parts of the manuscript. J.B. helped with mice work and data analysis, contributed to figure 8E. S.B. isolated monocytes for *in vitro* experiments, M.K. established settings for FACS and helped with data analysis. N.S. provided figure 1E. A.W., M.S., R.S.C., and A. K. provided human samples. C.S. performed LPS-experiment (data not shown). M.L. helped with data analysis for figure 6D. M.G.M. and H.M. provided reagents. A.A. suggested experiment shown in 6B, M.Pa. and G.v.L. provided LysMCre*Ccr2*$^{loxP/loxP}$ mice. M.Pr. provided Tie2CCR2/*Ccr2*$^{-/-}$ mice and figures 6D and 8A. L.B. suggested and planned experiments, helped with mice work and data analysis, wrote parts of the manuscript. M.H. suggested experiments, helped with data analysis and wrote parts of the manuscript.

Cancer Cell
Article

Endothelial CCR2 Signaling Induced by Colon Carcinoma Cells Enables Extravasation via the JAK2-Stat5 and p38MAPK Pathway

Monika Julia Wolf,[1,12] Alexandra Hoos,[4,12] Judith Bauer,[5] Steffen Boettcher,[2] Markus Knust,[6,7] Achim Weber,[3] Nicole Simonavicius,[5] Christoph Schneider,[8] Matthias Lang,[6] Michael Stürzl,[9] Roland S. Croner,[9] Andreas Konrad,[9] Markus G. Manz,[2] Holger Moch,[3] Adriano Aguzzi,[1] Geert van Loo,[10] Manolis Pasparakis,[11] Marco Prinz,[6] Lubor Borsig,[4,13,*] and Mathias Heikenwalder[1,5,13,*]

[1]Institute of Neuropathology
[2]Division of Hematology
[3]Institute of Surgical Pathology
University Hospital Zurich, CH-8091 Zurich, Switzerland
[4]Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, CH-8057 Zurich, Switzerland
[5]Institute of Virology, Technische Universität München/Helmholtz Zentrum München, D-81675 Munich, Germany
[6]Department of Neuropathology & BIOSS Centre for Biological Signaling Studies, University of Freiburg, D-79106 Freiburg, Germany
[7]Faculty of Biology, University of Freiburg, D-79104 Freiburg, Germany
[8]Institute of Integrative Biology, ETH Zurich, CH-8952 Schlieren, Switzerland
[9]Division of Molecular and Experimental Surgery, Department of Surgery, University Hospital Erlangen, D-91054 Erlangen, Germany
[10]Department for Molecular Biomedical Research, VIB Gent, B-9052 Gent-Zwijnaarde, Belgium
[11]Institute of Genetics, Centre for Molecular Medicine (CMMC) and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, D-50674 Cologne, Germany
[12]These authors contributed equally to this work
[13]These authors contributed equally to this work
*Correspondence: lborsig@access.uzh.ch (L.B.), heikenwaelder@helmholtz-muenchen.de (M.H.)
http://dx.doi.org/10.1016/j.ccr.2012.05.023

SUMMARY

Increased expression of the chemokine CCL2 in tumor cells correlates with enhanced metastasis, poor prognosis, and recruitment of CCR2$^+$Ly6C^{hi} monocytes. However, the mechanisms driving tumor cell extravasation through the endothelium remain elusive. Here, we describe CCL2 upregulation in metastatic UICC stage IV colon carcinomas and demonstrate that tumor cell-derived CCL2 activates the CCR2$^+$ endothelium to increase vascular permeability in vivo. CCR2 deficiency prevents colon carcinoma extravasation and metastasis. Of note, CCR2 expression on radio-resistant cells or endothelial CCR2 expression restores extravasation and metastasis in *Ccr2*$^{-/-}$ mice. Reduction of CCR2 expression on myeloid cells decreases but does not prevent metastasis. CCL2-induced vascular permeability and metastasis is dependent on JAK2-Stat5 and p38MAPK signaling. Our study identifies potential targets for treating CCL2-dependent metastasis.

INTRODUCTION

Metastasis, the spread of tumor cells to vital organs, is the leading cause of cancer-related death in humans (Gupta and Massagué, 2006). Understanding the mechanisms driving metastasis is therefore essential for developing new therapeutic strategies. Metastasis is a multistage process comprising tumor cell dissemination, survival in the circulation, extravasation, and ultimately colonization of distant organs (Chambers et al., 2002; Joyce and Pollard, 2009). Tumor cell extravasation, colonization

Significance

Metastasis is the primary cause of cancer-related mortality. Elevated expression of CCL2 attracts CCR2$^+$Ly6C^{hi} monocytes and enhances metastasis. We show that metastatic UICC stage IV colon carcinomas upregulate CCL2. Using both in vivo and in vitro models, we demonstrate that colon carcinoma-derived CCL2 activates endothelial cells through CCR2 and is dependent on JAK2-Stat5 and p38MAPK phosphorylation. Our results show that a tumor cell-derived chemokine induces vascular permeability and enables efficient tumor cell extravasation, suggesting a so-far undescribed role for chemokines in tumor cell extravasation. Moreover, we identified two targets for the suppression of CCL2-dependent tumor cell extravasation during colon carcinoma metastasis. Chemokine-dependent control of vascular permeability during metastasis is likely to occur in various cancers.

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

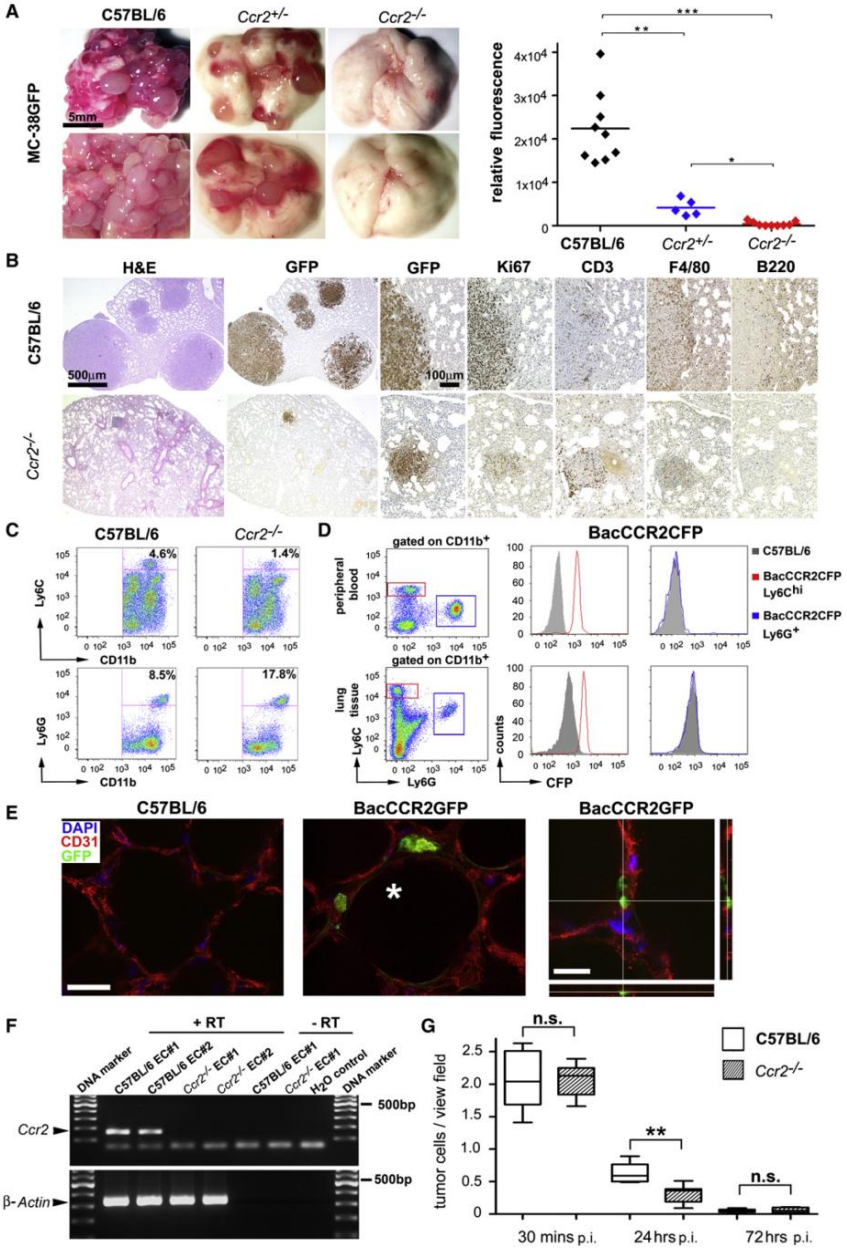


Figure 1. CCR2 Determines Susceptibility to Experimental Lung Metastasis in a Dose-Dependent Manner

(A) Macroscopy of lungs derived from C57BL/6 (n = 9), *Ccr2*$^{+/-}$ (n = 5), *Ccr2*$^{-/-}$ mice (n = 9) on day 28 p.i. with MC-38GFP cells. Size of scale bar is indicated (left panel). Quantification of tumor load by GFP fluorescence in lung homogenates (right panel).

(B) Histological analysis of colon carcinoma tumors (MC-38GFP) in C57BL/6 and *Ccr2*$^{-/-}$ lungs at 28 d.p.i. Low and high magnifications (scale bars) are indicated. H&E, hematoxylin and eosin; GFP, tumor cells; Ki67, proliferating cells; CD3, T cells; F4/80, macrophages, B220: B cells.

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

as well as outgrowth are considered to be the limiting steps in metastasis (Chambers et al., 2002). It has long been recognized that the tumor cell microenvironment, composed of fibroblasts, endothelial cells, and leukocytes, significantly contributes to metastatic dissemination (Joyce and Pollard, 2009). In particular, myeloid-derived monocytes/macrophages, commonly found in various types of malignant cancers were shown to facilitate tumor cell extravasation and metastatic outgrowth (Peinado et al., 2011; Qian and Pollard, 2010). Analysis of the metastasis-supporting niche revealed that soluble factors derived from the local environment and from tumors are responsible for mobilization of bone marrow (BM)-derived cells during metastasis (Peinado et al., 2011).

Chemokines and their receptors were found to be involved in metastasis and also to be direct targets of oncogene activation (Allavena et al., 2011; Hiratsuka et al., 2006). Stromal cells, infiltrating leukocytes and tumor cells themselves were identified as sources of cytokines and chemokines, both at primary tumors and metastatic sites (Läubli and Borsig, 2010; Mishra et al., 2011; O'Hayre et al., 2008). Highly metastatic cells have been shown to induce BM-derived macrophages to express cytokines and chemokines emphasizing the role of a reciprocal crosstalk of tumor cells with the microenvironment to actively shape the metastatic niche (Kim et al., 2009). Local activation of endothelia by metastasizing tumor cells induced CCL5 expression, which was associated with monocyte recruitment during the initial phase of metastasis. Accordingly, inhibition of CCL5 reduced metastasis (Läubli et al., 2009). Recently, CCL2 has been identified as the major factor facilitating breast cancer metastasis to the lung (Qian et al., 2011). Clinical evidence clearly associated elevated levels of CCL2 and CCL5 with poor prognosis in breast, colon, prostate, and cervix cancer patients due to metastatic progression (Soria et al., 2011; Yoshidome et al., 2009; Zhang et al., 2010; Zijlmans et al., 2006). Monocytes recruited to tumors through the CCL2-CCR2 axis can be polarized to an alternatively activated M2-phenotype thereby contributing to immunosuppression and enhanced tumor cell survival (Loberg et al., 2007; Mantovani and Sica, 2010). CCL2 has been shown to induce angiogenic activation of endothelial cells along with inflammatory responses (Salcedo et al., 2000), and CCL2-mediated recruitment of inflammatory monocytes promoted metastasis (Qian et al., 2011). Similarly, overexpression of CCL2 in PC-3 prostate cancer cells led to increased bone metastasis associated with elevated accumulation of macrophages (Mizutani et al., 2009). Consequently, CCL2-neutralizing antibody treatment significantly prolonged survival of tumor-bearing mice due to inhibition of metastasis (Lu and Kang, 2009; Mizutani et al., 2009; Qian et al., 2011; Salcedo et al., 2000). Although elevated CCL2 expression is clearly linked to metastasis through the recruitment of monocytes/macrophages, the exact mechanisms by which CCL2 signaling facilitates tumor cell extravasation at the endothelial barrier and subsequent metastatic colonization remain elusive.

Here, we investigate the role of the CCL2-CCR2 chemokine axis during metastatic dissemination and the involvement of the endothelium in this process.

RESULTS

CCR2 Promotes Metastasis of Colon Carcinoma Cells

To determine whether tumor cell extravasation and growth was altered in the absence of CCR2, C57BL/6 and *Ccr2*$^{-/-}$ mice were intravenously (i.v.) injected with syngeneic GFP$^+$ colon carcinoma cells (MC-38GFP) and lungs were macroscopically scored for the presence of metastatic foci 28 days postinjection (d.p.i.). *Ccr2*$^{-/-}$ lungs displayed fewer tumors than C57BL/6 lungs ($p < 0.001$; Figure 1A; Figure S1A available online). Development of tumors depended on CCR2 expression levels in the recipient host, since *Ccr2*$^{+/-}$ lungs contained fewer metastases than C57BL/6 ($p < 0.01$) but still more than *Ccr2*$^{-/-}$ ($p < 0.05$; Figures 1A, S1A, and S1B). This was corroborated by measurement of relative GFP fluorescence in lung homogenates (Figure 1A). Immunohistochemistry revealed no obvious differences in the relative composition of Ki67$^+$, CD3$^+$, F4/80$^+$, and B220$^+$ cells within the tumors (Figure 1B).

Next, we investigated whether differences in the immune cell composition could influence metastasis. No differences in numbers of CD4$^+$ or CD8$^+$ T cells, CD19$^+$ B cells, NK1.1$^+$, or F4/80$^+$ cells could be identified between naive C57BL/6 and *Ccr2*$^{-/-}$ lungs by flow cytometry (Figure S1C). However, *Ccr2*$^{-/-}$ lungs displayed reduced numbers of CD11b$^+$Ly6C^{hi}Ly6G$^-$ monocytes ($p < 0.001$; denoted as Ly6C^{hi}) and a relative increase in CD11b$^+$Ly6G$^+$ cells ($p < 0.01$; Figures 1C, S1D, and S1E). To identify which cells express CCR2, we performed flow cytometry on blood and lung tissue from BacCCR2CFP reporter mice, expressing CFP under the CCR2 promoter (Hohl et al., 2009). Almost all CD11b$^+$Ly6C^{hi} cells were CFP positive, whereas CD11b$^+$Ly6G$^+$, CD19$^+$, CD4$^+$, and CD8$^+$ cells were CFP negative (Figures 1D and S1F). We next investigated whether nonhematopoietic cells in lungs express CCR2. Confocal microscopy of BacCCR2GFP mice revealed that CD31$^+$ lung endothelial cells expressed GFP (Figures 1E and S1G), as confirmed by transcriptional analysis of purified primary endothelial cells from C57BL/6 and *Ccr2*$^{-/-}$ lungs (Figures 1F, S1I, and S1J).

Altered numbers of colon carcinoma cells in lungs of *Ccr2*$^{-/-}$ mice could explain the observed inability to form tumors. However, no differences in the amount and distribution of

(C) Flow cytometry analysis of monocytes in naive lungs of *Ccr2*$^{-/-}$ and C57BL/6 mice.
(D) Flow cytometry of CFP$^+$ cells in blood and lung tissue of BacCCR2CFP transgenic mice shows that most Ly6C^{hi} (red) but no Ly6G (blue) cells express CFP.
(E) Confocal microscopy analysis on lung tissue of C57BL/6 and BacCCR2GFP mice for the expression of GFP. Endothelial cells are positive for GFP (green) and CD31 (red); nuclei are stained in blue (DAPI). The asterisk indicates the alveolar space; scale bars: 20 μm (low magnification); 15 μm (high magnification); Z-stacks are indicated.
(F) RT-PCR of *Ccr2* expression in CD31-sorted primary endothelial cells isolated from lungs of C57BL/6 and *Ccr2*$^{-/-}$ mice. Two samples for each genotype are shown (+RT), including controls without reverse transcriptase treatment (-RT). *β-actin* served as control (bp, base pairs).
(G) Time course analysis of tumor cell survival in lung tissue. Numbers of tumor cells/view field were analyzed on sections of C57BL/6 and *Ccr2*$^{-/-}$ lungs 30 min, 24 hr and 72 hr p.i. (n = 3, each; mean with min/max is shown). Statistics: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n.s., not significant.
See also Figure S1.

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

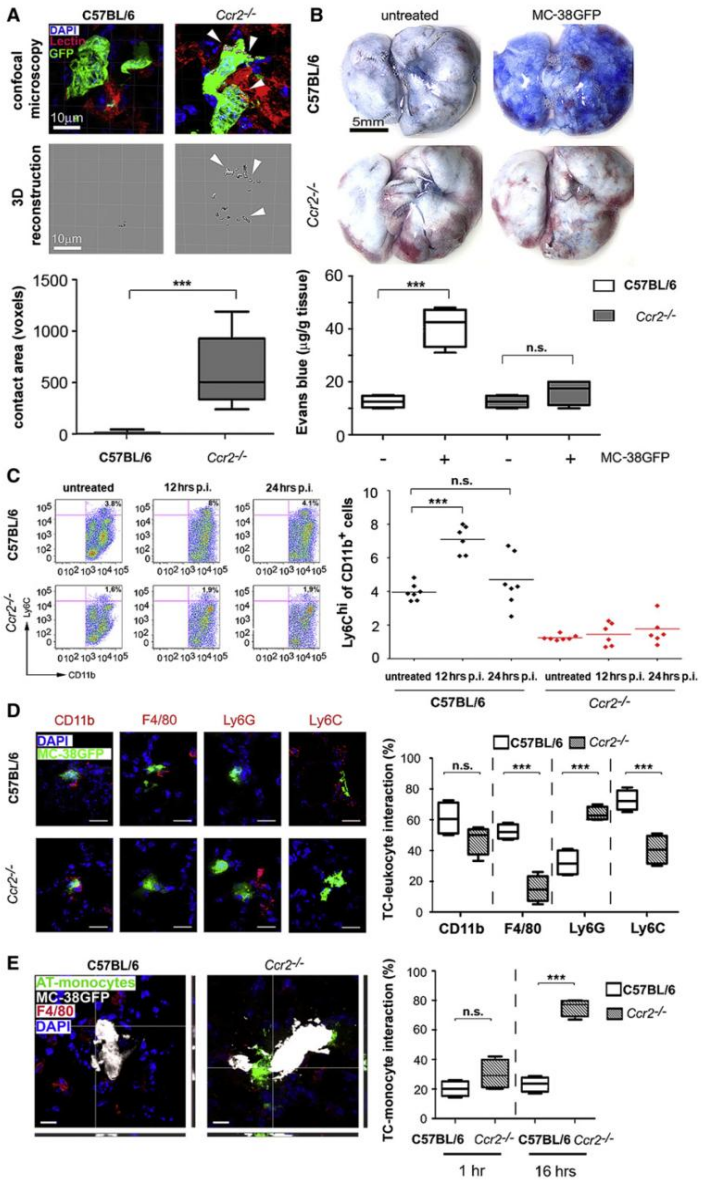


Figure 2. CCR2 Deficiency Reduces Tumor Cell Extravasation and Vascular Permeability and Affects Myeloid-Tumor Cell Interaction

(A) Confocal microscopy of MC-38GFP cells (green) and endothelial cells (red; lectin$^+$) in C57BL/6 and *Ccr2*$^{-/-}$ lungs (n = 5) 24 hr p.i. with MC-38GFP cells (upper row). Size of scale bar is indicated. Three-dimensional reconstruction of the interaction of both cell types (lower row). Quantification of the 3D contact area in voxels is shown as percentiles.

(B) Macroscopy of naive C57BL/6 and *Ccr2*$^{-/-}$ lungs (upper row) as well as 24 hr p.i. with MC-38GFP cells (lower row) upon Evans blue administration. Size of scale bar is indicated. Spectrophotometric quantification of Evans blue extracted from C57BL/6 and *Ccr2*$^{-/-}$ lungs (n = 6, each).

(C) Flow cytometry analysis for CD11b$^+$Ly6C^{hi} cells in lung tissue upon tumor cell challenge of C57BL/6 and *Ccr2*$^{-/-}$ mice over time (left panel). Quantification of Ly6C^{hi} cells is presented in the right panel (n = 6–7).

(D) Confocal microscopy images of the interaction of myeloid cells (CD11b, F4/80, Ly6G, Ly6C; all stained in red) with MC-38GFP cells (green) in C57BL/6 and *Ccr2*$^{-/-}$ mice 24 hr p.i. are shown. Nuclei are stained in blue (DAPI; left panel). Quantification of the myeloid-tumor cell interaction in lungs; percentiles are indicated (n = 3, each). Scale bar: 20 μm (right panel).

(E) Confocal microscopy analysis of the interaction of adoptively transferred, PKH26-labeled monocytes (green) with MC-38GFP cells (white) and endogenous F4/80$^+$ macrophages (red) in C57BL/6 and *Ccr2*$^{-/-}$ mice (n = 3) 16 hr p.i. (left panel). Scale bar: 10 μm; Z-stacks are indicated. Quantification of myeloid-tumor cell interaction in lungs; percentiles are indicated (right panel; mean with min/max). Statistics: ***p < 0.001; n.s., not significant.

See also Figure S2.

MC-38GFP cells in the lung vasculature were found in either genotype as early as 30 min p.i. (Figure 1G). At later time points (24, 72 hr), numbers of MC-38GFP cells in lung tissue decreased in both genotypes, though slightly more pronounced in *Ccr2*$^{-/-}$ mice at 24 hr. Besides, no significant changes were found in the structure and organization of the CD31$^+$ alveolar vasculature in both genotypes (Figure S1H).

CCR2 Controls Endothelial Permeability and Tumor Cell Extravasation

Reduced lung metastasis in *Ccr2*$^{-/-}$ mice could be explained by an impaired ability of MC-38GFP cells to extravasate into parenchyma of *Ccr2*$^{-/-}$ lungs. Confocal microscopy revealed that MC-38GFP cells remained associated with tomato lectin-stained endothelium of blood vessels in *Ccr2*$^{-/-}$ lungs at 24 hr p.i. (Figure 2A). In contrast, there was minimal contact of MC-38GFP cells with endothelia in C57BL/6 mice at 24 hr p.i., indicating that tumor cells had already extravasated from blood vessels. Three-dimensional reconstruction of confocal images showed increased contact of MC-38GFP cells with endothelium in *Ccr2*$^{-/-}$ lungs compared to C57BL/6 (p < 0.001; Figure 2A). To determine whether reduced capacity of MC-38GFP cells to extravasate in *Ccr2*$^{-/-}$ lungs was due to decreased vascular permeability, we

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

tested the ability of Evans blue to permeate lung tissue. No Evans blue leakage occurred in naive lungs of either genotype, indicating full vascular integrity (Figure 2B). However, 24 hr p.i. with MC-38GFP cells C57BL/6 lungs readily took up Evans blue, whereas *Ccr2*$^{-/-}$ lungs remained white, indicating no increase in vascular permeability (Figure 2B). Quantification of Evans blue confirmed the macroscopic data ($p < 0.001$; Figures 2B and S2C). The increase in vascular permeability upon injection of MC-38GFP cells was transient, as revealed by time course analyses (Figures S2A and S2B).

Next, the hypothesis whether reduced capacity of MC-38GFP cells to extravasate in *Ccr2*$^{-/-}$ lungs correlates with reduced recruitment of leukocytes was tested (Läubli et al., 2006; Qian et al., 2011). Flow cytometry of lungs from mice injected with MC-38GFP cells revealed recruitment of Ly6C^{hi} cells in C57BL/6 mice that was strongly reduced in *Ccr2*$^{-/-}$ mice (Figure 2C). The increase of Ly6C^{hi} cells in C57BL/6 lungs persisted for approximately 12–24 hr p.i. No significant increase of other immune cell types (e.g., CD4$^+$, CD8$^+$, CD11c$^+$, NK1.1$^+$) was found (data not shown).

In addition, we investigated whether lung-infiltrating myeloid cell populations are recruited to MC-38GFP cells at sites of vascular arrest. F4/80$^+$, CD11b$^+$, and Ly6C$^+$ cells associated less with tumor cells in *Ccr2*$^{-/-}$ lungs compared to C57BL/6. In contrast, increased association of Ly6G$^+$ cells with MC-38GFP cells was observed in *Ccr2*$^{-/-}$ lungs ($p < 0.001$; Figure 2D).

Finally, to test whether MC-38GFP cells injected into *Ccr2*$^{-/-}$ mice would still efficiently recruit myeloid cells, we adoptively transferred myeloid cells to mice 6 hr post-tumor cell injection. MC-38GFP cells recruited transferred myeloid cells in *Ccr2*$^{-/-}$ but not in C57BL/6 lungs ($p < 0.001$; Figure 2E) as MC-38GFP cells in C57BL/6 lungs had already recruited endogenous myeloid cells.

CCR2 Expression on Radio-Resistant Cells Enables Efficient Metastasis

We next tested whether reconstitution of *Ccr2*$^{-/-}$ mice with CCR2$^+$ BM cells could restore the ability of MC-38GFP cells to extravasate and metastasize into lungs. Reciprocal BM reconstitutions (C57BL/6 → *Ccr2*$^{-/-}$; *Ccr2*$^{-/-}$ → C57BL/6) and controls (C57BL/6 → C57BL/6; *Ccr2*$^{-/-}$ → *Ccr2*$^{-/-}$) were performed and blood was analyzed for the presence of CCR2$^+$Ly6C^{hi} monocytes 6–8 weeks after reconstitution (Figures 3A and S3A). MC-38GFP cells were administered to reconstituted mice (efficiency >90%) and analyzed for tumor growth in lungs (Figures 3A and S3B). C57BL/6 → C57BL/6 mice displayed robust metastasis similar to control C57BL/6 mice, whereas *Ccr2*$^{-/-}$ → *Ccr2*$^{-/-}$ mice lacked or had strongly reduced metastasis. CCR2 expression on radio-resistant cells (*Ccr2*$^{-/-}$ → C57BL/6) resulted in more metastasis when compared to mice devoid of CCR2 expression in the stromal compartment (C57BL/6 → *Ccr2*$^{-/-}$; $p < 0.05$). This indicates that both, hematopoietic and stromal CCR2 expression is required for efficient metastasis.

To delineate the role of stromal CCR2 expression during metastasis, we assessed whether endothelial cell-restricted CCR2 expression (i.e., Tie2CCR2/*Ccr2*$^{-/-}$ mice; (Mildner et al., 2009) would enable tumor cell extravasation and metastasis. Tie2CCR2/*Ccr2*$^{-/-}$ mice lacked Ly6C^{hi} monocytes in blood and lung tissue and lacked CCR2 expression on CD11b$^+$, CD19$^+$ or CD3$^+$ cells in blood, spleen and BM (Figures 3B and S3C). Transcriptional analysis indicated *Ccr2* expression in whole lung tissue and in purified lung endothelial cells in Tie2CCR2/*Ccr2*$^{-/-}$ mice (Figures S3D and S3E). Upon injection with MC-38GFP cells, tumor cell extravasation and metastasis was partially restored in Tie2CCR2/*Ccr2*$^{-/-}$ compared to C57BL/6 mice (Figure 3C). Quantification of metastatic foci, GFP fluorescence and immunohistochemistry confirmed these data (Figures 3C, 3D, and S3F). Hence, expression of CCR2 on endothelial cells was sufficient to partially restore metastasis.

CCR2 Expression on Myeloid Cells Contributes to Tumor Cell Metastasis

To assess the role of CCR2 on myeloid cells during metastasis, we bred *Ccr2*$^{loxP/loxP}$ with *LysMCre* mice (Clausen et al., 1999), resulting in mice with reduced CCR2 expression on myeloid cells. Similar amounts of Ly6C^{hi} monocytes were detected in blood from *LysMCreCcr2*$^{loxP/loxP}$ and C57BL/6 mice by flow cytometry (Figure 4A).

We next challenged *LysMCreCcr2*$^{loxP/loxP}$ mice with MC-38GFP cells and quantified Ly6C^{hi} monocytes in lungs at 6 and 12 hr p.i. (Figures 4B and S4A). Strong reduction (>90%) in the influx of Ly6C^{hi} monocytes to the lungs and a decrease in local recruitment of F4/80$^+$ and CD11b$^+$ cells to tumor cells were observed (Figure 4C). To determine the effect of reduced CCR2$^+$/Ly6C^{hi} monocyte recruitment on metastasis, *LysMCreCcr2*$^{loxP/loxP}$, *Ccr2*$^{loxP/loxP}$, *Ccr2*$^{-/-}$, and C57BL/6 mice were injected with MC-38GFP and analyzed 28 d.p.i. Lung metastasis was increased in *LysMCreCcr2*$^{loxP/loxP}$ compared to *Ccr2*$^{-/-}$ mice ($p < 0.05$; Figures 4D and S4B). However, in comparison to C57BL/6 mice, metastasis in *LysMCreCcr2*$^{loxP/loxP}$ mice was decreased. This was confirmed by quantification of GFP fluorescence and immunohistochemistry of lung tissues (Figures 4D and S4C). Thus, CCR2 expression on myeloid cells contributes to metastasis of MC-38GFP cells.

Tumor Cell-Derived CCL2 Expression Controls Myeloid Cell Recruitment

Whether CCR2-dependent lung metastasis occurs also with different tumor cells, we injected mice with Lewis lung carcinoma cells (3LL). Similar to MC-38GFP cells, attenuation of metastasis was observed in *Ccr2*$^{-/-}$ when compared to C57BL/6 lungs ($p < 0.05$; Figure 5A, upper panels). Next, we injected B16-BL6 melanoma cells. Of note, similar extent of lung metastasis was observed in C57BL/6 and *Ccr2*$^{-/-}$ mice indicating that B16-BL6 melanoma cells extravasate and metastasize independently of CCR2 (Figure 5A, lower panels).

The dependency of metastasis on host-derived CCR2 expression indicates the involvement of tumor cell-intrinsic factors. We therefore first compared chemokine and chemokine receptor *mRNA* expression levels in lungs of C57BL/6 and *Ccr2*$^{-/-}$ mice injected with MC-38GFP cells. A strong increase in *Ccl2*, *Ccl7*, *Ccl12*, *Cxcl1*, and *Cxcl10* expression was detected at 4 hr p.i. in C57BL/6 lungs (Figure 5B). These transcripts remained abundant 8 and 12 hr p.i. and decreased at 48 hr p.i. (Figure 5B and data not shown). Similar transcriptional changes occurred in lungs of MC-38GFP-injected *Ccr2*$^{-/-}$ mice (Figure 5B), suggesting that chemokine induction does not depend on host-derived

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

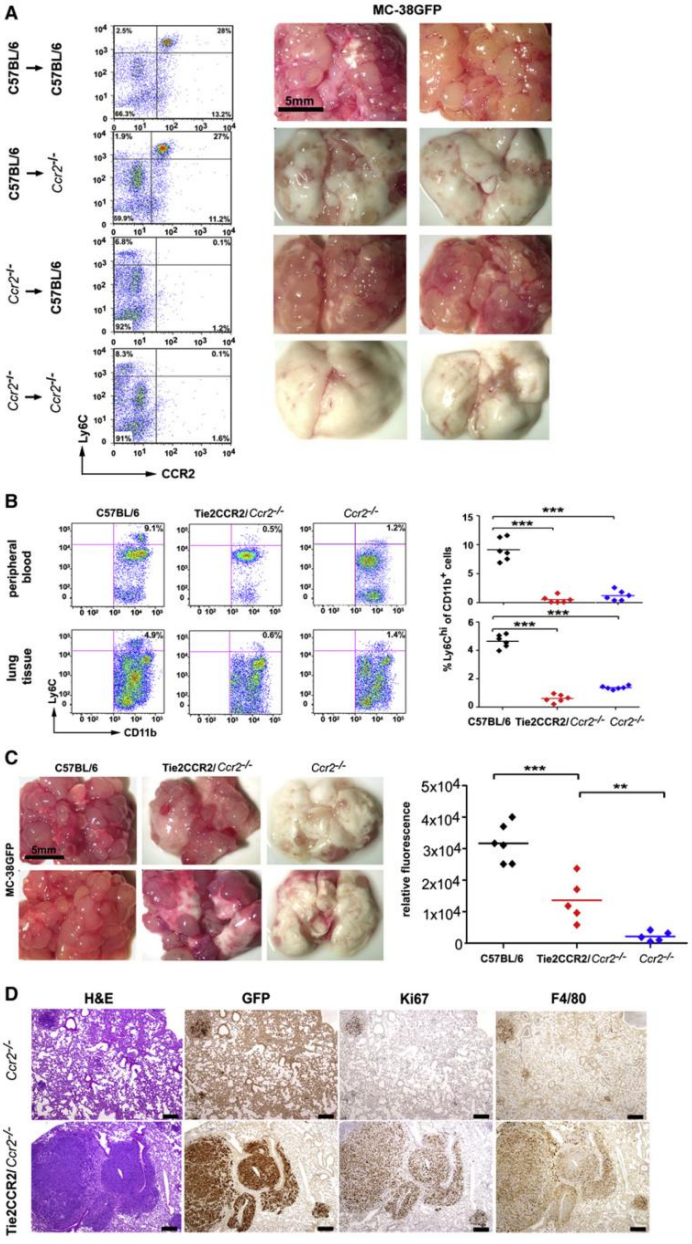


Figure 3. CCR2 Expression on Radio-Resistant Cells Suffices for Tumor Cell Metastasis

(A) Flow cytometry analysis of blood from C57BL/6→C57BL/6 (n = 8), C57BL/6→*Ccr2*$^{-/-}$ (n = 9), *Ccr2*$^{-/-}$→C57BL/6 (n = 7) and *Ccr2*$^{-/-}$→*Ccr2*$^{-/-}$ (n = 5) chimeric mice for the presence of CCR2^{+} Ly6C^{hi} cells (left row). Macroscopy of lungs from MC-38GFP-injected chimeric mice 28 d.p.i. Size of scale bar is indicated (middle/ right row).

(B) Flow cytometry for CD11b^{+}Ly6C^{hi} cells in blood (upper row) and lungs (lower row) of naive C57BL/6, Tie2CCR2/*Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice (left panel). Quantification of Ly6C^{hi} cells is presented in the right panel (n = 6, each).

(C) Macroscopy of lungs from MC-38GFP-injected C57BL/6, Tie2CCR2/*Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice 28 d.p.i. Size of scale bar is indicated (left panel). Quantification of GFP fluorescence in lung homogenates of C57BL/6, Tie2CCR2/*Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice (n = 5, each; right panel).

(D) Histological analysis of MC-38GFP tumors in lungs of Tie2CCR2/*Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice. H&E: Hematoxylin/Eosin, GFP: tumor cells, Ki67: proliferating cells, F4/80: macrophages. Scale bar: 50 μm; statistics: ***$p < 0.001$; **$p < 0.01$.

See also Figure S3.

CCR2. Alternatively, tumor cells may directly cause increased chemokine expression by altering the cellular composition and/or activation status of cells in the lungs of MC-38GFP-injected mice. However, chemokine receptor *mRNA* expression was unaltered in lungs of C57BL/6 and *Ccr2*$^{-/-}$ mice p.i. (Figure 5C). When we compared chemokine expression in MC-38GFP, 3LL, and B16-BL6 cells, elevated levels of *Ccl2*, *Ccl7*, *Cxcl1*, and *Cxcl10* transcripts were detected in MC-38GFP tumor cells compared to C57BL/6 colon (Figure S5A). Of note, *Ccl2 mRNA* and protein levels were elevated in MC-38GFP and 3LL cells and low in B16-BL6 melanoma cells, indicating that CCR2-dependent metastasis relies on tumor cell-derived CCL2 (Figures 5D, S5B, and S5C).

To determine whether MC-38 and 3LL-derived CCL2 was required for tumor cell extravasation and metastasis, CCL2 expression was silenced with small hairpin RNA (shRNA) in MC-38GFP cells (MC-38GFP^{CCL2kd}). *Ccl2 mRNA* was reduced by 75%–90%, CCL2 protein was reduced by 60%–85%, and expression of other chemokines/cytokines remained unaffected (Figure 5D). Cells stably transduced with scrambled shRNA served as control (MC-38GFP^{scr}). MC-38GFP and MC-38GFP^{CCL2kd} cells were injected into C57BL/6 mice, and their ability to recruit Ly6C^{hi} monocytes to the lung at 4 and 12 hr p.i. was assayed by flow cytometry. Similar numbers of Ly6C^{hi} cells were detected at 4 hr p.i. in lungs of C57BL/6 mice injected with MC-38GFP^{CCL2kd} or MC-38GFP cells (Figure 5E). However, no specific recruitment of Ly6C^{hi} monocytes

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

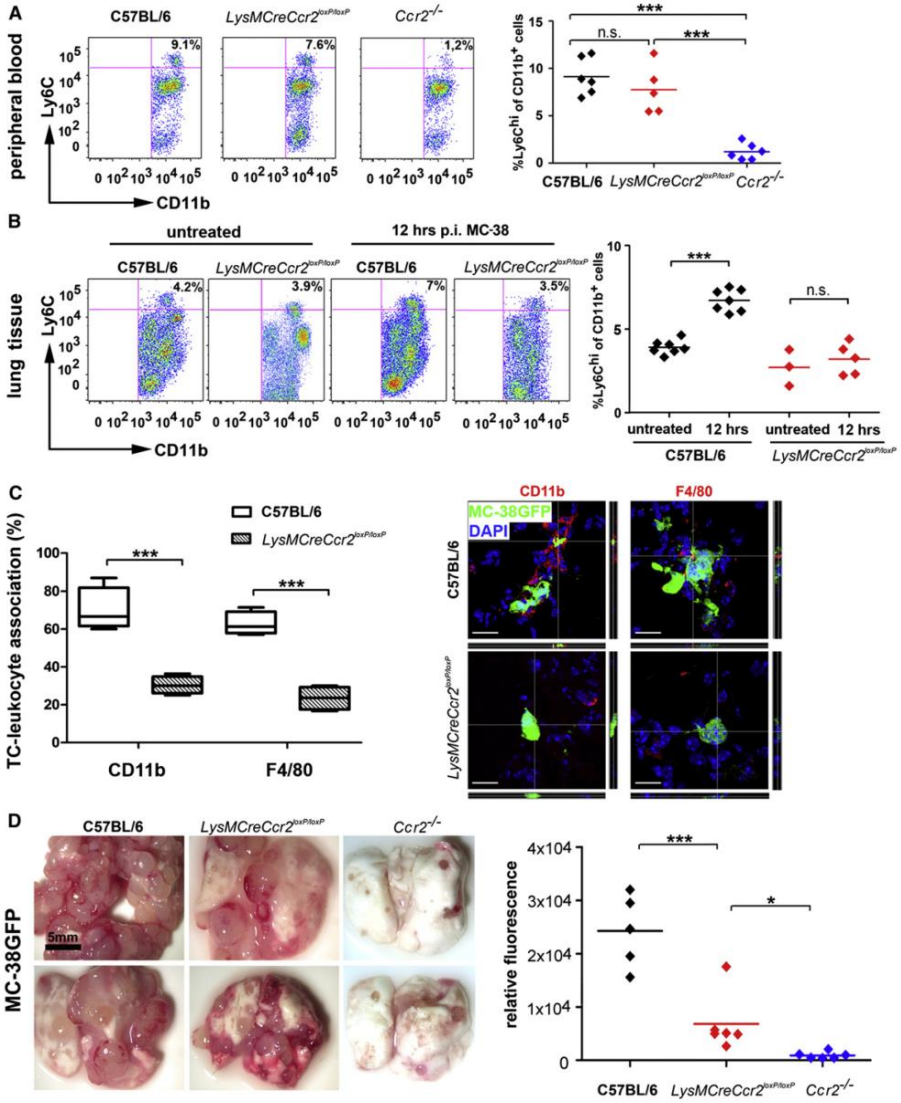


Figure 4. CCR2 Expression on Ly6C^{hi} Monocytes Facilitates Tumor Cell Extravasation

(A) Flow cytometry for CD11b^{+}Ly6C^{hi} cells in blood of C57BL/6, *LysMCreCcr2*$^{loxP/loxP}$ and *Ccr2*$^{-/-}$ mice (n = 5–6; left panel). Quantification of Ly6C^{hi} cells (right panel).

(B) Flow cytometry for CD11b^{+}Ly6C^{hi} cells in lungs of naive C57BL/6 and MC-38-injected C57BL/6 and *LysMCreCcr2*$^{loxP/loxP}$ mice 12 hr p.i. (n = 5–7; left panel). Quantification of Ly6C^{hi} cells (right panel).

(C) Confocal microscopy investigating the interaction of myeloid cells (CD11b, F4/80; red) and tumor cells (green) in lungs of C57BL/6 and *LysMCreCcr2*$^{loxP/loxP}$ mice. Nuclei are stained in blue (DAPI); scale bar: 20 μm; Z-stacks are indicated.

(D) Macroscopy of lungs from C57BL/6, *LysMCreCcr2*$^{loxP/loxP}$ and *Ccr2*$^{-/-}$ mice 28 d.p.i. with MC-38GFP. Size of scale bar is indicated (left panel). Quantification of GFP fluorescence in lung homogenates of C57BL/6 (n = 5), *LysMCreCCR2*$^{loxP/loxP}$ (n = 6), and *Ccr2*$^{-/-}$ (n = 6) mice (right panel). Statistics: ***$p < 0.001$; *$p < 0.05$; n.s., not significant.

See also Figure S4.

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

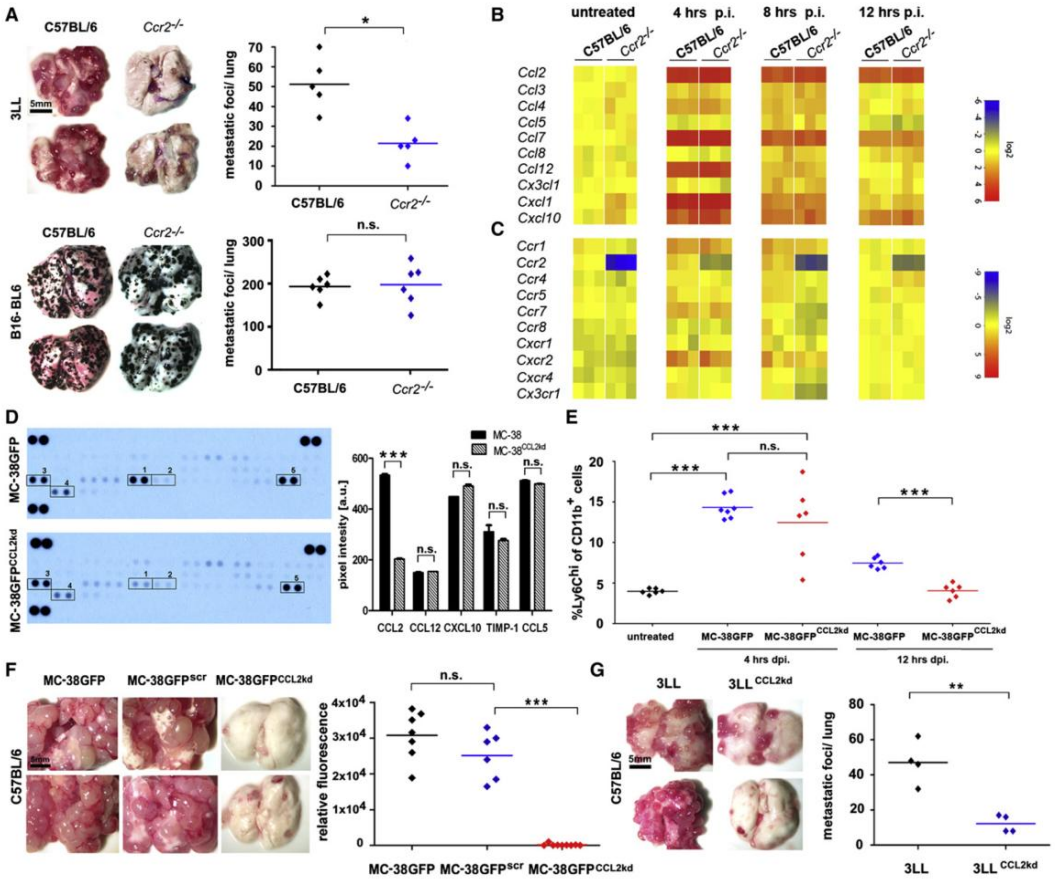


Figure 5. Tumor Cell-Derived CCL2-Dependent and -Independent Mechanisms of Tumor Cell Extravasation

(A) Macroscopy of lungs from C57BL/6 and *Ccr2*$^{-/-}$ mice at 12 d.p.i. with Lewis lung carcinoma cells (3LL) and quantification of tumor nodules (n = 5, each; upper row). Macroscopy of lungs from C57BL/6 and *Ccr2*$^{-/-}$ mice at 14 d.p.i. with melanoma cells (B16-BL6) and quantification of tumor numbers (n = 6, each; lower row).

(B and C) Real-time PCR analysis for the expression of selected chemokines (B) and chemokine receptors (C) in lungs of C57BL/6 and *Ccr2*$^{-/-}$ mice. Untreated, 4 hr p.i., 8 hr p.i., and 12 hr p.i. with MC-38GFP are shown from left to right. Data are presented as ΔΔct values in a log2 scale (red: upregulated; blue: down-regulated). Columns indicate individual mice (n = 3); rows represent particular genes. Each data point reflects the median expression of a particular gene resulting from three to four technical replicates, normalized to the mean expression value of the respective gene in C57BL/6 lungs.

(D) Expression profile of various chemokines and cytokines in MC-38GFP and MC-38GFP^{CCL2kd} cells. 1: CCL2 (silenced); 2: CCL12; 3: CXCL10; 4: TIMP-1; 5: CCL5 remain unaffected. Dots in the upper right and left corners serve as loading controls (left panel). Quantification of pixel density (shown in arbitrary units [a.u.]; mean ± SEM; right panel).

(E) Quantification of CD11b^{+}Ly6C^{hi} cells in lungs of naive C57BL/6 mice and 4 hr and 12 hr p.i. with MC-38GFP and MC-38GFP^{CCL2kd} cells (n = 6–7).

(F) Macroscopy of lungs derived from C57BL/6 mice 28 d.p.i. with MC-38GFP, MC-38GFP^{scr} and MC-38GFP^{CCL2kd} cells. Size of scale bar is indicated. Quantification of GFP fluorescence in lung homogenates of C57BL/6 mice 28 d.p.i. with MC-38GFP (n = 7), MC-38GFP^{scr} (n = 6) and MC-38GFP^{CCL2kd} (n = 9) cells.

(G) Macroscopy of lungs from C57BL/6 mice 12 d.p.i. with 3LL and 3LL^{CCL2kd} cells. Size of scale bar is indicated (left panel). Quantification of tumor nodules (n = 4, each; right panel); statistics: ***p < 0.001; **p < 0.01; *p < 0.05; n.s., not significant.

See also Figure S5.

was detected in lungs of C57BL/6 mice 12 hr p.i. with MC-38GFP^{CCL2kd} cells. Therefore, specific recruitment of Ly6C^{hi} cells is controlled by MC-38-derived CCL2. Next, we analyzed tumor cell extravasation and metastasis in C57BL/6 mice injected with MC-38GFP^{CCL2kd}, MC-38GFP or MC-38GFP^{scr} cells. Reduced lung metastasis was observed in C57BL/6 mice upon

MC-38GFP^{CCL2kd} injection and confirmed by analysis of GFP fluorescence ($p < 0.001$; Figure 5F). Similar results were obtained by silencing of CCL2 in 3LL cells ($p < 0.01$; Figure 5G).

In addition, reduced CCL2 expression in MC-38GFP cells (MC-38GFP^{CCL2kd}) diminished the ability to interact with CD11b$^+$, F4/80$^+$, or Ly6C$^+$ cells ($p < 0.001$). Although MC-38GFP^{CCL2kd} cells showed enhanced interaction with Ly6G$^+$ cells ($p < 0.001$; Figure S5D), they failed to recruit adoptively transferred myeloid cells (Figure S5E). This supports the conclusion that tumor cell-derived CCL2 is required for the association of tumor cells with myeloid cells.

CCR2 Expression on Endothelial Cells Determines Lung Permeability

To determine whether endothelial CCR2 expression was sufficient to induce vascular permeability, we injected Tie2CCR2/*Ccr2*$^{-/-}$ mice with MC-38GFP cells. Elevated levels of Evans blue in Tie2CCR2/*Ccr2*$^{-/-}$ lungs suggest that endothelial CCR2 activation is sufficient to induce lung vascular permeability (Figure 6A). Since host-derived CCL2 has been implicated in metastasis (Qian et al., 2011), we injected *Ccl2*$^{-/-}$ mice with MC-38GFP cells. Interestingly, lung vascular permeability was increased upon tumor cell injection ($p < 0.001$; Figure 6A), indicating that tumor cells induce vascular permeability in the absence of host-derived CCL2. In line, MC-38GFP^{CCL2kd} cells failed to induce vascular permeability in C57BL/6 lungs (Figure 6A). To investigate whether the local microenvironment (e.g., CCL2 expression; monocytes; stromal cells) rescues the inability of CCL2-deficient tumor cells to extravasate and metastasize, we coinjected MC-38 and MC-38GFP^{CCL2kd} cells into C57BL/6 mice. Similar numbers of metastases were observed in lungs of C57BL/6 mice injected with MC-38 versus MC-38GFP^{CCL2kd}/MC-38 cells 28 d.p.i. ($p = 0.6$; Figure 6B). Of note, lungs from C57BL/6 mice injected with MC-38GFP^{CCL2kd}/MC-38 cells displayed mainly GFP-negative tumors, suggesting that tumors mainly originated from CCL2$^+$ tumor cells ($p < 0.001$; Figures 6B and 6C). Therefore, the inability of MC-38GFP^{CCL2kd} cells to metastasize cannot be restored by the local environment.

We next examined lungs from C57BL/6 and *Ccr2*$^{-/-}$ mice 12 hr p.i. with MC-38 cells at ultrastructural level. Injection of MC-38 cells induced changes in C57BL/6 lung tissue including increased thickness of airway epithelial cells in the bronchi and thickened smooth muscle cells. Of note, tumor cells were found inside the alveoli (Figure 6D, left panel) and rarely in the vessels. Alveoli in C57BL/6 lungs appeared shrunken, with numerous alveolar macrophages and type I, II pneumocytes. Moreover, we observed an intimate interaction between MC-38 cells and C57BL/6 endothelia with tumor cell protrusions spanning through the apical side of the endothelium resembling ongoing tumor cell transmigration (Figure 6D, middle panel, inset). In contrast, *Ccr2*$^{-/-}$ lung endothelium appeared to be less affected by the injection of MC-38 tumor cells with no visible indication for endothelial attachment (Figure 6D, right panel, inset).

Endothelial CCR2 Signaling Controls Tumor Cell Extravasation through the JAK2-Stat5 and p38MAPK Pathways

To identify the mechanisms involved in tumor cell extravasation, we next examined transmigration of tumor cells through lung endothelial monolayers in presence or absence of monocytes in vitro. MC-38GFP cells incubated on C57BL/6 endothelial cells for 16 hr transmigrated only minimally toward an FCS gradient. Cocultivation of MC-38GFP cells with BM-derived monocytes induced efficient transmigration of MC-38GFP cells through C57BL/6 endothelia ($p < 0.001$; Figure 7A). In contrast, MC-38GFP cells were unable to transmigrate through endothelial cells isolated from *Ccr2*$^{-/-}$ mice either in the presence or absence of monocytes ($p = 0.6$; Figure 7A). To determine whether tumor cell transmigration depends on tumor cell-derived CCL2, we tested the ability of MC-38GFP^{CCL2kd} cells to transmigrate in vitro. MC-38GFP^{CCL2kd} cells could not transmigrate through a C57BL/6 endothelial monolayer in the presence of monocytes ($p < 0.001$; Figure 7B). Interestingly, neither lack of endothelial nor monocytic CCR2 expression affected efficacy of monocyte transmigration (Figure 7C). Taken together, endothelial CCR2 signaling can specifically enable transmigration of tumor cells without affecting monocytes. Tumor cell-derived CCL2 was sufficient to induce permeability in the CCR2$^+$ endothelial monolayer even in the absence of monocytes. In contrast, MC-38GFP^{CCL2kd} cells induced partial endothelial permeability only in the presence of monocytes (Figure 7D). These findings provide direct evidence for the role of endothelial CCR2 activation during tumor cell extravasation; and for the supportive role of monocytes in this process.

CCL2 is known to activate Janus kinase 2 (JAK2) through CCR2 (Mellado et al., 1998), thereby triggering several downstream pathways such as Stat1, Stat3 and Stat5, p38MAPK, and PI3K (Agrawal et al., 2011; Sanz-Moreno et al., 2011). We first tested whether inhibition of JAK2 phosphorylation would affect tumor cell transmigration. AG490 effectively blocked tumor cell transmigration, demonstrating the requirement of CCR2-JAK2 signaling for tumor cell extravasation ($p < 0.001$; Figure 7E). Inhibition of Stat3 phosphorylation (S3I-201) failed to affect tumor cell migration ($p = 0.8$), while block of Stat5 phosphorylation impeded transmigration of MC-38GFP cells ($p < 0.001$; Figure 7E). PI3K inhibition with Wortmannin did not alter tumor cell transmigration ($p = 0.6$), whereas blocking p38MAPK phosphorylation with SB202190 did ($p < 0.001$, Figure 7F). Therefore, both JAK2-Stat5 and p38MAPK pathways appeared to be involved in transmigration of MC-38GFP cells through CCR2$^+$ endothelium.

We next addressed whether tumor cells, rather than transmigrating through endothelial junctions, might trans-invade endothelial cells (Feng et al., 1998), which was shown to depend on Rac/Rho GTPases. However, inhibition of Rac1 (with NSC23766) failed to block tumor cell transmigration pointing at transmigration through endothelial junctions (Figure 7E).

To provide further evidence that CCR2-JAK2 signaling is involved in tumor cell extravasation, we measured JAK2 phosphorylation in lung homogenates from MC-38-injected mice. Increased JAK2 phosphorylation relative to total JAK2 was observed at 8 and 12 hr p.i. in C57BL/6 lungs which was prevented by AG490. In contrast, inhibition of p38MAPK did not affect JAK2 phosphorylation (Figure 7F). Importantly, no or minor phosphorylation of JAK2 was detected in *Ccr2*$^{-/-}$ mice upon tumor cell injection, confirming that CCR2 expression in lungs is crucial for activation of this signaling cascade. Similarly, p38MAPK and Stat5 were activated in lungs of C57BL/6 mice

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

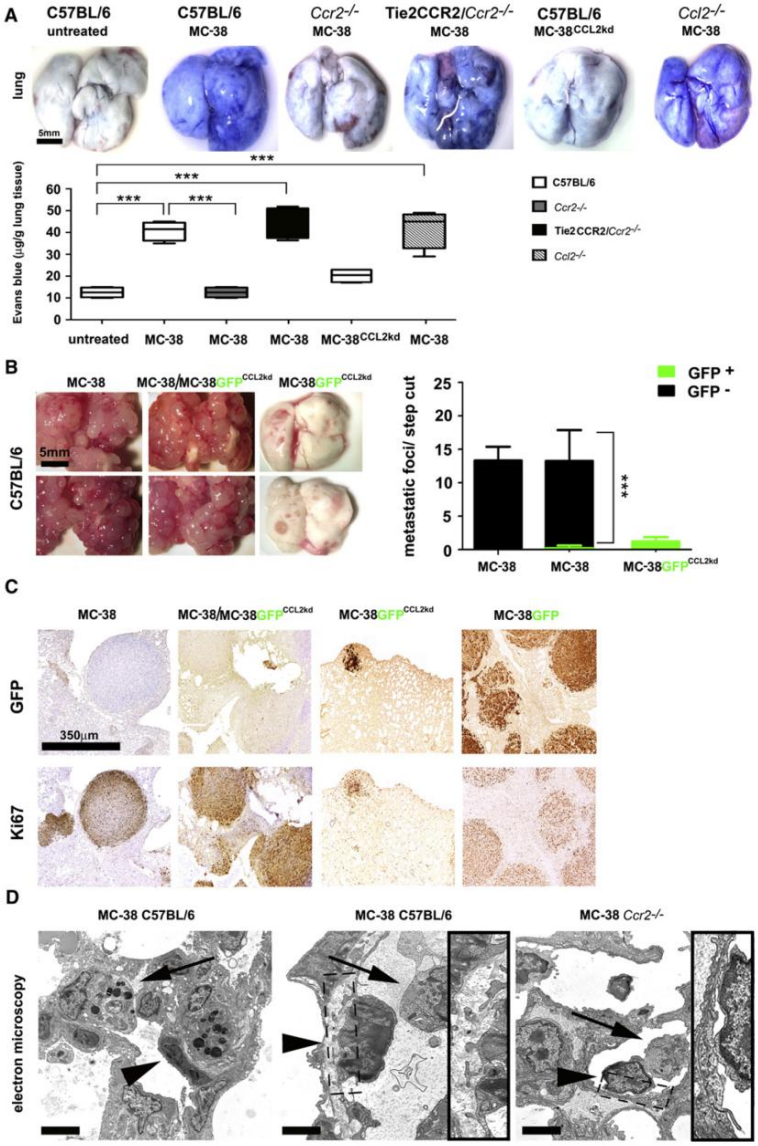


Figure 6. Tumor Cell-Derived CCL2 Is Sufficient to Induce Endothelial Permeability

(A) Macroscopy of lungs with Evans blue accumulation derived from untreated C57BL/6 mice or treated mice of various genotypes 24 hr p.i. with MC-38GFP and MC-38GFP^{CCL2kd} cells. Scale bar is indicated. Spectrophotometric quantification of Evans blue extracted from lung tissue.

(B) Macroscopy of lungs derived from C57BL/6 mice 28 d.p.i. with MC-38 (n = 3), MC-38/MC-38GFP^{CCL2kd} (1:1 ratio; n = 5) and MC-38GFP^{CCL2kd} cells (n = 3; left panel). Quantification of tumor nodules on step cuts through lungs (black bars represent MC-38 cells, green bars MC-38GFP^{CCL2kd} cells; mean ± SEM; right panel).

(C) Immunohistochemistry for the quantification of tumor nodules. GFP and Ki67 served as tumor markers. Size of scale bar is indicated.

(D) Electron microscopy images of lungs of C57BL/6 (left and middle) and *Ccr2*$^{-/-}$ mice (right) 12 hr p.i. with MC-38GFP. Left: arrowhead points toward a tumor cell in the alveolar space, in close contact to a type II pneumocyte (arrow). Middle panel: arrowhead shows an attached and transmigrating tumor cell localized within the vessel. The arrow indicates a monocyte within the vessel. The insert shows the interaction between tumor cell and vessel wall at higher magnification. Right: No visible transmigration of tumor cells (arrow head) in lungs of *Ccr2*$^{-/-}$ mice. Arrow: monocyte within the vessel. Scale bar: 5 μm; statistics: ***p < 0.001.

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

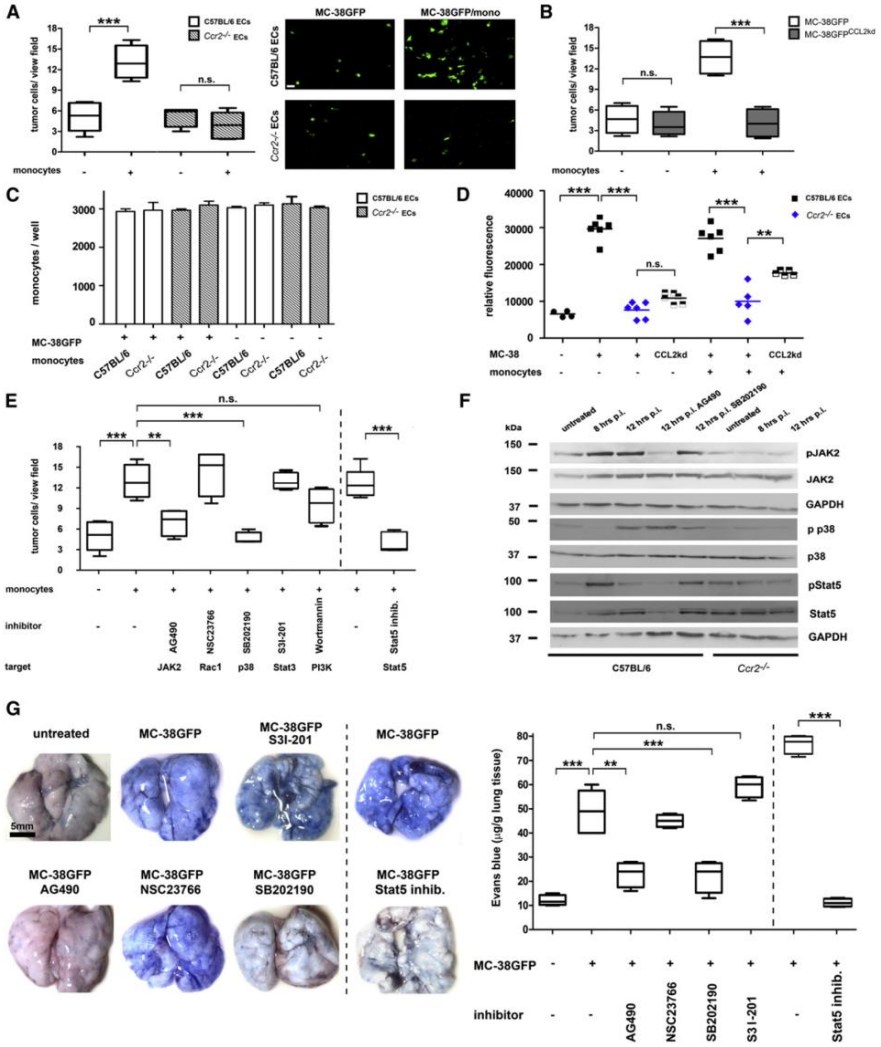


Figure 7. CCL2-CCR2-Mediated Permeabilization of the Endothelium Is Driven by JAK2-Stat5 and p38MAPK activation

(A) MC-38GFP cells were cocultured with monocytes for 16 hr on monolayers of endothelial cells derived either from C57BL/6 or *Ccr2*$^{-/-}$ lungs. Transmigrated GFP^{+} tumor cells in the lower chamber were counted and are presented as percentiles (left panel). Representative images of transmigrated tumor cells for each coculture; scale bar: 10 μm (right panel).

(B) MC-38GFP cells were compared with MC-38GFP^{CCL2kd} cells for their efficiency to migrate through C57BL/6 endothelial cells. Transmigrated GFP^{+} tumor cells were counted and values are presented as percentiles.

(C) Analysis of transmigrated monocytes at 16 hr post coculture with C57BL/6 or *Ccr2*$^{-/-}$ endothelial cells with or without tumor cells (mean ± SEM).

(D) Permeability of the C57BL/6 or *Ccr2*$^{-/-}$ endothelial cell layer for Dextran-FITC was determined at 7 hr post addition of tumor cells with or without monocytes.

(E) Inhibitors for JAK2 (AG490), Rac (NSC23766), p38MAPK (SB201290), Stat3 (S3I-201), PI3K (Wortmannin), and Stat5 pathways were added to the coculture of MC-38GFP cells with monocytes. GFP^{+} tumor cells transmigrated through the C57BL/6 endothelial cell layer were counted in the lower chamber.

(F) Immunoblot analysis of lung samples derived from C57BL/6 and *Ccr2*$^{-/-}$ mice (untreated, 8 and 12 hr p.i.) as well as C57BL/6 mice treated with AG490 or SB202190 (12 hr p.i.). Panel from top to bottom: pJAK2, JAK2, GAPDH, P-p38, p38, pStat5, Stat5, and GAPDH.

(G) Macroscopy of lungs upon Evans blue administration in presence or absence of inhibitors for Stat3, JAK2, Rac1, p38MAPK and Stat5 16 hr p.i. with MC-38GFP cells. Quantification of Evans blue (n = 4, each; right panel; mean with min/max is shown). Statistics: ***p < 0.001; **p < 0.01; n.s., not significant.

See also Figure S6.

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

upon tumor cell injection (between 8 and 12 hr p.i.) but not in *Ccr2*$^{-/-}$ lungs (Figure 7F).

We next tested the involvement of the above-described signaling pathways in controlling lung permeability. In line with our in vitro data, JAK2, Stat5 and p38MAPK inhibition prevented the increase in vascular permeability induced by MC-38GFP cells in C57BL/6 mice. However, treatment with Rac1 (NSC23766) and Stat3 (S3I-201) inhibitor failed to affect vascular permeability (Figure 7G). Since a specific inhibitor of PI3K did not affect tumor cell transmigration in vitro, it is unlikely that PI3K signaling is involved in tumor cell extravasation. Accordingly, PI3K$\gamma^{-/-}$ mice developed lung metastasis similar to C57BL/6 mice 28 d.p.i. of MC-38GFP cells (data not shown). Thus, inhibition of JAK2-Stat5 and p38MAPK signaling prevents tumor cell-induced vascular permeability in vivo.

We next investigated whether JAK2-Stat5 or p38MAPK inhibition exclusively affects endothelial cells or also monocytes. We therefore analyzed levels of phosphorylated Stat5 and p38MAPK in MC-38GFP-injected Tie2CCR2/*Ccr2*$^{-/-}$ mice (Figures S6A and S6B). Increase in phosphorylation of JAK2, Stat5 and p38MAPK was comparable both in Tie2CCR2/*Ccr2*$^{-/-}$ and C57BL/6 lungs. Therefore, Stat5 and p38MAPK activation in endothelial cells occurs in the absence of Ly6C^{hi} monocytes.

We next determined whether lack of CCR2 signaling in vivo would affect the expression of Stat5 and p38MAPK target genes associated with the vascular integrity of endothelial cells. Significantly decreased expression of *E-selectin* and *Icam-1* ($p < 0.05$; $p < 0.001$) was found in *Ccr2*$^{-/-}$ lungs compared to C57BL/6 at 8 hr p.i. (Figure S6C). This suggested that the proinflammatory endothelial response associated with metastasis was reduced in *Ccr2*$^{-/-}$ mice. Aside from these transcriptional changes, we observed that coincubation of primary endothelial cells with MC-38 cells caused cytoskeletal retraction and disruption of the endothelial layer, as determined by phalloidin-fluorescein isothiocyanate (FITC) staining (Figure S6D).

We next investigated on ultrastructural level, whether the inhibition of CCR2 signaling affects tumor cell behavior in lungs of C57BL/6 mice. In contrast to lungs of naive mice, but similar to C57BL/6 tumor injected mice, thickened airway epithelial cells and smooth muscle cells were found in mice treated with JAK2 inhibitor. However, strongly reduced tumor cell extravasation could be observed in AG490 treated mice (Figure 8A).

Finally, we assessed whether JAK2 and p38MAPK inhibition would also block lung metastasis. Thus, we treated MC-38GFP-injected mice with AG490 or SB202190 during the first 3 d.p.i. Blockade of both, JAK2 and p38MAPK signaling attenuated metastasis ($p < 0.05$; Figures 8B and 8C).

CCL2 Expression Correlates with Metastatic Potential in Human Colon Cancer Tissue

We next analyzed *CCL2* expression in human primary nonmetastasized colon tumors (UICC stages I and II) and in colon tumors that metastasized into the lymph nodes (UICC stage) or into distant organs (UICC stage IV). *CCL2* transcripts were more abundant in primary colon tumors of stages I, II, and III when compared to healthy colon samples. However, *CCL2* expression was particularly high in colon tumors stage IV that developed metastases in distant organs (Figure 8D), indicating that upregulation of *CCL2* correlates with metastatic potential.

DISCUSSION

During the multistep process of metastasis, cytokines and chemokines have been reported to have pro- or antitumorigenic effects (Granot et al., 2011; Qian et al., 2011). Elevated CCL2 levels have been previously linked to malignancy and increased metastasis in a number of cancers (Soria et al., 2011; Yoshidome et al., 2009; Zhang et al., 2010; Zijlmans et al., 2006). Our analysis of primary colon tumors (UICC stages I–IV) confirmed the link between CCL2 upregulation in stage IV colon carcinoma and metastatic capacity. Recent studies have shown that monocyte recruitment by CCL2 contributes to lung metastasis of breast cancer (Lu and Kang, 2009; Qian et al., 2011). Our data provide evidence that tumor cell-derived CCL2 activates CCR2 on endothelial cells, thereby enabling efficient tumor cell extravasation.

To study the mechanisms of tumor cell extravasation, we applied chimeric, transgenic, and knockout mice, as well as in vitro assays. This allowed us to define the role of tumor cell-derived CCL2 in a spatial and temporal manner. Using these models, we determined that enhanced lung vascular permeability, tumor cell extravasation, and recruitment of Ly6C^{hi} monocytes are initiated by tumor cell-derived CCL2 at sites of vascular arrest in a CCR2-dependent manner.

It was previously demonstrated that systemic depletion of CCL2 with neutralizing antibodies could attenuate metastasis, while the origin of CCL2 was identified to be in tumor cells and stromal compartment (Qian et al., 2011). We show that vascular permeability was enhanced in both *Ccl2*$^{-/-}$ and C57BL/6 mice, suggesting that host-derived CCL2 is not required. In addition, silencing of CCL2 expression in two different tumor cell lines (MC-38GFP and 3LL) prevented induction of lung vascular permeability and subsequent metastasis, further demonstrating that tumor cell-derived CCL2 is sufficient for initiation of tumor cell extravasation. Previous observations that CCL2 overexpression in tumor cells enhanced metastasis are in line with our findings (Lu and Kang, 2009). Importantly, recent work indicated that CCR2 deficiency does not affect primary tumor growth (Sawanobori et al., 2008), arguing against the possibility that the observed reduction in metastasis is the result of tumor growth rate.

Which cellular compartment integrates tumor cell-derived CCL2 signaling through CCR2? CCL2-dependent recruitment of monocytes to metastatic sites has been shown to contribute to metastasis (Mizutani et al., 2009; Qian et al., 2011). It is also known that CCL2-expressing breast tumor cells engage CCR2^{+} cells of monocytic origin to facilitate colonization of lung and bone (Lu and Kang, 2009). Consistent with this, we show that recruitment of Ly6C^{hi} monocytes correlates with metastasis. However, depletion of CCR2 from monocytes in *LysMCreCcr2*$^{loxP/loxP}$ mice strongly affected monocyte recruitment and interaction with tumor cells and consequently reduced but did not prevent metastasis. In line, even partial reduction of CCR2 affected monocyte recruitment (Leuschner et al., 2011). Accordingly, the number of metastases in *Ccr2*$^{-/-}$→C57BL/6 chimeric mice was considerably lower than in C57BL/6 mice. Furthermore, experiments with Tie2CCR2/*Ccr2*$^{-/-}$ mice confirmed that endothelial expression of CCR2 is sufficient for metastasis (Figure 3C). Therefore, Ly6C^{hi} monocytes appear to be necessary but not sufficient for effective metastasis.

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

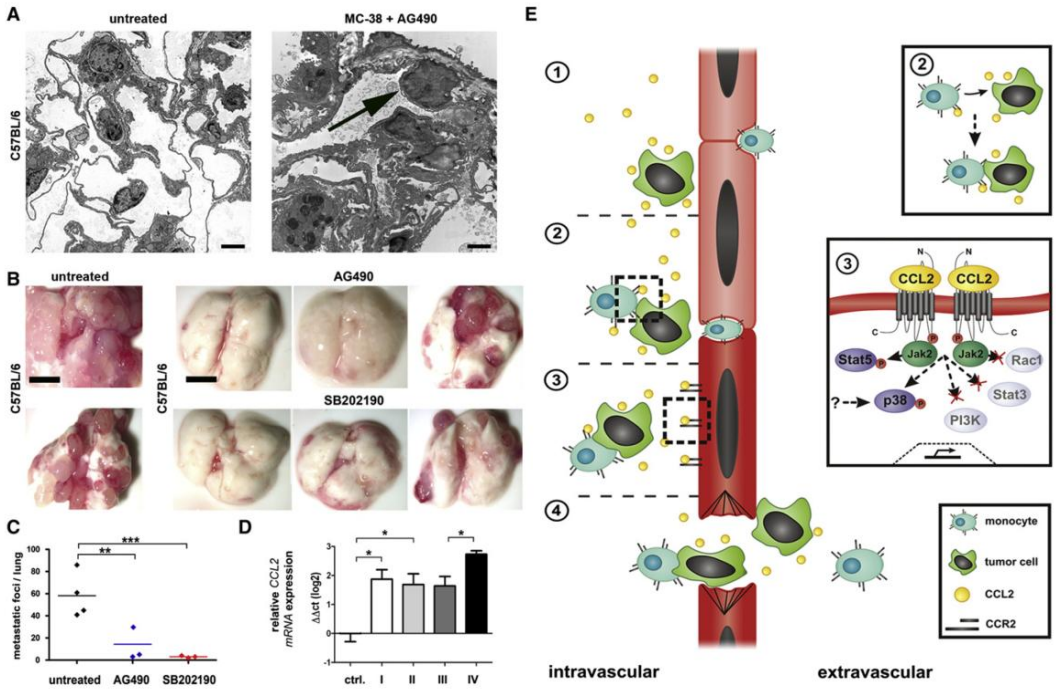


Figure 8. Short-Term Inhibition of JAK2 or p38MAPK Impedes Tumor Cell Metastasis

(A) Electron microscopy images of a naive C57BL/6 lung (left, scale bar: 4 μm) and 12 hr p.i. with MC-38GFP and AG490 treatment (right, scale bar: 2 μm); arrow points toward a tumor cell.

(B) Macroscopy of lungs from C57BL/6 mice 26 d.p.i. with MC-38GFP left untreated or with AG490 or SB202190 treatment during the first 3 d.p.i. Scale bar: 5 mm.

(C) Quantification of tumor nodules in lungs of C57BL/6 mice left untreated (n = 4), SB202190 (n = 3), or AG490 (n = 3) treated upon MC-38GFP injection.

(D) Transcriptional analysis of *CCL2* levels in tissue obtained from colon-cancer patients. *mRNA* expression levels in healthy control tissue (ctrl.; n = 4), in tumor samples classified as stage I (n = 10), stage II (n = 10) both being nonmetastatic; stage III: metastastic in lymph nodes (n = 10), stage IV: metastatic in distant organs (n = 9) colon carcinomas were studied. Data are presented in a log2 scale. Each bar reflects the median expression (mean ± SEM) of a gene resulting from three to four technical replicates, normalized to the mean expression value of *CCL2* in control samples. Statistics: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

(E) Schematic model depicting how CCL2-expressing tumor cells attract monocytes (1 and 2), trigger vascular permeability (3) and transmigrate through the endothelium (4). Tumor cell-dependent activation of CCR2 on the endothelium induces JAK2, Stat5, and p38MAPK phosphorylation but not PI3K, Stat3, and Rac1.

Here, we demonstrate that endothelial CCR2 signaling controls metastasis by promoting tumor cell extravasation. We hypothesize that CCR2 on endothelial cells may resemble a "lock-and-key" signal for opening the vasculature and enabling extravasation of $CCL2^+$ tumor cells. Indeed, we found that expression of CCR2 on endothelial cells is linked to the induction of vascular permeability. This "lock-and-key" relationship was dependent on CCL2 expression solely from the tumor cells, since MC-38^{CCL2kd} cells failed to induce vascular permeability and metastasis in C57BL/6 mice. Since coinjection experiments of MC-38GFP^{CCL2kd}/MC-38 cells only induced efficient metastasis of $CCL2^+$ but not MC-38GFP^{CCL2kd} cells, we suggest that an intimate interaction of $CCL2^+$ colon carcinoma cells with the $CCR2^+$ endothelium is required.

Monocyte transmigration still occurred through CCR2-deficient endothelial monolayers, indicating that CCR2 signaling on endothelial cells specifically enables transmigration of $CCL2^+$ tumor cells.

There are several signaling pathways that participate in endothelial activation associated with diapedesis of leukocytes during inflammation (McIntyre et al., 2003), some of which act downstream of CCR2 (e.g., Stat3, PI3K (Yu et al., 2009). From the panel of inhibitors tested in vitro, we identified the JAK2, Stat5, and p38MAPK signaling pathways as responsible for induction of vascular permeability and extravasation in vivo. Activation of the p38MAPK in endothelial cells by transmigrating tumor cells has been observed previously (Tremblay et al., 2006). The pattern of JAK2 and p38MAPK activation in MC-38-injected C57BL/6 mice suggests that these two pathways act independently of each other, since inhibition of p38MAPK phosphorylation did not block Stat5 or JAK2 phosphorylation. In contrast, JAK2 inhibition blocked Stat5 but not p38MAPK

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

phosphorylation. Tumor cell-induced phosphorylation of both JAK2-Stat5 and p38MAPK was detected in Tie2CCR2/*Ccr2*$^{-/-}$ lungs, arguing that both signaling pathways are activated in parallel in the endothelial compartment.

Based on our data, we propose the following model describing the role of CCR2 signaling in metastasis: Upon vascular arrest, CCL2^{+} tumor cells induce a local chemokine gradient, recruiting CCR2^{+} monocytes. Concomitantly or subsequently direct activation of CCR2 on the endothelium is triggered by tumor cells, which is critical for metastasis. CCL2 activates JAK2 and p38MAPK signaling, leading to enhanced vascular permeability that along with monocyte recruitment enables efficient tumor cell extravasation (Figure 8E). The exact kinetics and modes of transmigration remain to be described. Our data identify a yet undescribed role for tumor cell-derived chemokines in metastasis that goes beyond the attraction of inflammatory cells.

With increasing interest, chemokines and chemokine receptors are being considered as targets for cancer therapy, including metastasis. Certainly, the CCL2-CCR2 axis is just one possible chemokine-chemokine receptor axis exploited by tumor cells. Nevertheless, further studies will be required to identify which cancers use chemokine-chemokine receptor interactions for efficient tumor cell extravasation and metastasis. Our results identify inhibition of CCR2 and its downstream targets (JAK2/Stat5/p38MAPK) as a potential strategy for preventing CCL2-mediated metastasis therapeutically.

EXPERIMENTAL PROCEDURES

Mice

Animals were maintained under specific pathogen-free conditions, and experiments were approved by Zürich Cantonal Veterinary Committee in accordance to the guidelines of the Swiss Animal Protection Law. C57BL/6 and *Ccl2*$^{-/-}$ mice were purchased from the Jackson Laboratory, *Ccr2*$^{-/-}$ mice (Boring et al., 1997; Kuziel et al., 1997) were either purchased from the Jackson Laboratory or obtained from our own breedings; Tie2CCR2/*Ccr2*$^{-/-}$ were described previously (Mildner et al., 2009); *LysMCreCcr2*$^{loxP/loxP}$ were obtained from M. Pasparakis (G.v.L. and M.P., unpublished data); and BacCCR2GFP and BacCCR2DTCFP mice (Hohl et al., 2009) were obtained from E. Pamer.

Human Colon Cancer Tissue

Patients with colon carcinomas stage UICC I (n = 10), II (n = 10), III (n = 10), and IV (n = 9) were selected from the Erlangen Registry for Colorectal Carcinomas (ERCRC). The local ethics committee approved the study and the regulations of the same committee of the clinical center Erlangen were obeyed; written consent has been obtained (approval nr. 3914).

Experimental Metastasis Assay

Mice were i.v. injected with MC-38GFP cells (3×10^5) and euthanized after 28 days. Metastatic foci were counted, macroscopic pictures of lungs were taken and GFP fluorescence was measured in lung homogenates (Borsig et al., 2002).

Vascular Permeability Assay

Permeability of the lung microvasculature was determined with Evans blue dye extravasation technique (Reutershan et al., 2006). Briefly, mice were injected with tumor cells, and, after 24 hr, 2 mg of Evans blue was i.v. injected followed by euthanasia 30 min later. In experiments using various signaling pathways inhibitors (AG490 and SB202190 [Sigma], NSC23766 [Calbiochem], S3I-201 and Stat5 [Santa Cruz]), inhibitors were i.p. injected 1 hr before and 5 hr post tumor cell injection at concentrations of 10–25 mg/kg. Lungs were perfused with PBS, dissected, photographed and homogenized. Evans blue was extracted by incubation with formamide at 60°C for 18 hr. Evans blue concentration was measured spectrophotometrically (absorbance at 620 nm).

Statistical Analysis

Statistical analysis was performed with the GraphPad Prism software (version 4.0). All data are presented as mean ± SEM and were analyzed by ANOVA with the post hoc Bonferroni multiple comparison test, unless specified differently. Analysis of two samples was performed with Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.05.023.

ACKNOWLEDGMENTS

We would like to thank Drs. Anna Lorentzen, Tracy O'Connor, Barbara Stecher, and Prof. Percy Knolle for critically reading the manuscript, Frank Tacke for mice, Yannick Böge, Renaud Maire, Robin Nagel, Silvia Behnke, Jay Tracy, Daniel Kull, Ruth Hillermann, Alexandra Müller, and Prof. Ursus Riede for support. M.H. was supported by an ERC Starting grant (LiverCancerMechanisms), the Helmholtz foundation, the Hofschneider foundation, Oncosuisse, and the Swiss National Foundation (no. 310030-130822). L.B. was supported by Swiss National Foundation (no. 31003A-133025). M.P. was supported by the center of chronic immunodeficiency (CCI) and the DFG (SFB 620, FOR1336, PR 577/8-1). M.S. and R.S.C. were supported by the German Federal Ministry for Education and Research.

Received: October 10, 2011
Revised: January 31, 2012
Accepted: May 18, 2012
Published: July 9, 2012

REFERENCES

Agrawal, S., Gollapudi, S., Su, H., and Gupta, S. (2011). Leptin activates human B cells to secrete TNF-α, IL-6, and IL-10 via JAK2/STAT3 and p38MAPK/ERK1/2 signaling pathway. J. Clin. Immunol. *31*, 472–478.

Allavena, P., Germano, G., Marchesi, F., and Mantovani, A. (2011). Chemokines in cancer related inflammation. Exp. Cell Res. *317*, 664–673.

Boring, L., Gosling, J., Chensue, S.W., Kunkel, S.L., Farese, R.V., Jr., Broxmeyer, H.E., and Charo, I.F. (1997). Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. J. Clin. Invest. *100*, 2552–2561.

Borsig, L., Wong, R., Hynes, R.O., Varki, N.M., and Varki, A. (2002). Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc. Natl. Acad. Sci. USA *99*, 2193–2198.

Chambers, A.F., Groom, A.C., and MacDonald, I.C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nat. Rev. Cancer *2*, 563–572.

Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Förster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. *8*, 265–277.

Feng, D., Nagy, J.A., Pyne, K., Dvorak, H.F., and Dvorak, A.M. (1998). Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. J. Exp. Med. *187*, 903–915.

Granot, Z., Henke, E., Comen, E.A., King, T.A., Norton, L., and Benezra, R. (2011). Tumor entrained neutrophils inhibit seeding in the premetastatic lung. Cancer Cell *20*, 300–314.

Gupta, G.P., and Massagué, J. (2006). Cancer metastasis: building a framework. Cell *127*, 679–695.

Hiratsuka, S., Watanabe, A., Aburatani, H., and Maru, Y. (2006). Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. Nat. Cell Biol. *8*, 1369–1375.

Cancer Cell
Tumor Cell-Derived CCL2 Controls Metastasis

Hohl, T.M., Rivera, A., Lipuma, L., Gallegos, A., Shi, C., Mack, M., and Pamer, E.G. (2009). Inflammatory monocytes facilitate adaptive CD4 T cell responses during respiratory fungal infection. Cell Host Microbe *6*, 470–481.

Joyce, J.A., and Pollard, J.W. (2009). Microenvironmental regulation of metastasis. Nat. Rev. Cancer *9*, 239–252.

Kim, S., Takahashi, H., Lin, W.W., Descargues, P., Grivennikov, S., Kim, Y., Luo, J.L., and Karin, M. (2009). Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature *457*, 102–106.

Kuziel, W.A., Morgan, S.J., Dawson, T.C., Griffin, S., Smithies, O., Ley, K., and Maeda, N. (1997). Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. Proc. Natl. Acad. Sci. USA *94*, 12053–12058.

Läubli, H., and Borsig, L. (2010). Selectins promote tumor metastasis. Semin. Cancer Biol. *20*, 169–177.

Läubli, H., Stevenson, J.L., Varki, A., Varki, N.M., and Borsig, L. (2006). L-selectin facilitation of metastasis involves temporal induction of Fut7-dependent ligands at sites of tumor cell arrest. Cancer Res. *66*, 1536–1542.

Läubli, H., Spanaus, K.S., and Borsig, L. (2009). Selectin-mediated activation of endothelial cells induces expression of CCL5 and promotes metastasis through recruitment of monocytes. Blood *114*, 4583–4591.

Leuschner, F., Dutta, P., Gorbatov, R., Novobrantseva, T.I., Donahoe, J.S., Courties, G., Lee, K.M., Kim, J.I., Markmann, J.F., Marinelli, B., et al. (2011). Therapeutic siRNA silencing in inflammatory monocytes in mice. Nat. Biotechnol. *29*, 1005–1010.

Loberg, R.D., Ying, C., Craig, M., Day, L.L., Sargent, E., Neeley, C., Wojno, K., Snyder, L.A., Yan, L., and Pienta, K.J. (2007). Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. Cancer Res. *67*, 9417–9424.

Lu, X., and Kang, Y. (2009). Chemokine (C-C motif) ligand 2 engages CCR2+ stromal cells of monocytic origin to promote breast cancer metastasis to lung and bone. J. Biol. Chem. *284*, 29087–29096.

Mantovani, A., and Sica, A. (2010). Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr. Opin. Immunol. *22*, 231–237.

McIntyre, T.M., Prescott, S.M., Weyrich, A.S., and Zimmerman, G.A. (2003). Cell-cell interactions: leukocyte-endothelial interactions. Curr. Opin. Hematol. *10*, 150–158.

Mellado, M., Rodríguez-Frade, J.M., Aragay, A., del Real, G., Martín, A.M., Vila-Coro, A.J., Serrano, A., Mayor, F., Jr., and Martínez-A, C. (1998). The chemokine monocyte chemotactic protein 1 triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. J. Immunol. *161*, 805–813.

Mildner, A., Mack, M., Schmidt, H., Brück, W., Djukic, M., Zabel, M.D., Hille, A., Priller, J., and Prinz, M. (2009). CCR2+Ly-6Chi monocytes are crucial for the effector phase of autoimmunity in the central nervous system. Brain *132*, 2487–2500.

Mishra, P., Banerjee, D., and Ben-Baruch, A. (2011). Chemokines at the crossroads of tumor-fibroblast interactions that promote malignancy. J. Leukoc. Biol. *89*, 31–39.

Mizutani, K., Sud, S., McGregor, N.A., Martinovski, G., Rice, B.T., Craig, M.J., Varsos, Z.S., Roca, H., and Pienta, K.J. (2009). The chemokine CCL2 increases prostate tumor growth and bone metastasis through macrophage and osteoclast recruitment. Neoplasia *11*, 1235–1242.

O'Hayre, M., Salanga, C.L., Handel, T.M., and Allen, S.J. (2008). Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. Biochem. J. *409*, 635–649.

Peinado, H., Lavotshkin, S., and Lyden, D. (2011). The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. Semin. Cancer Biol. *21*, 139–146.

Qian, B.Z., and Pollard, J.W. (2010). Macrophage diversity enhances tumor progression and metastasis. Cell *141*, 39–51.

Qian, B.Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L.R., Kaiser, E.A., Snyder, L.A., and Pollard, J.W. (2011). CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature *475*, 222–225.

Reutershan, J., Morris, M.A., Burcin, T.L., Smith, D.F., Chang, D., Saprito, M.S., and Ley, K. (2006). Critical role of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. J. Clin. Invest. *116*, 695–702.

Salcedo, R., Ponce, M.L., Young, H.A., Wasserman, K., Ward, J.M., Kleinman, H.K., Oppenheim, J.J., and Murphy, W.J. (2000). Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood *96*, 34–40.

Sanz-Moreno, V., Gaggioli, C., Yeo, M., Albrengues, J., Wallberg, F., Viros, A., Hooper, S., Mitter, R., Féral, C.C., Cook, M., et al. (2011). ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. Cancer Cell *20*, 229–245.

Sawanobori, Y., Ueha, S., Kurachi, M., Shimaoka, T., Talmadge, J.E., Abe, J., Shono, Y., Kitabatake, M., Kakimi, K., Mukaida, N., and Matsushima, K. (2008). Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. Blood *111*, 5457–5466.

Soria, G., Ofri-Shahak, M., Haas, I., Yaal-Hahoshen, N., Leider-Trejo, L., Leibovich-Rivkin, T., Weitzenfeld, P., Meshel, T., Shabtai, E., Gutman, M., and Ben-Baruch, A. (2011). Inflammatory mediators in breast cancer: coordinated expression of TNFα & IL-1β with CCL2 & CCL5 and effects on epithelial-to-mesenchymal transition. BMC Cancer *11*, 130.

Tremblay, P.L., Auger, F.A., and Huot, J. (2006). Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of p38 and ERK MAP kinases. Oncogene *25*, 6563–6573.

Yoshidome, H., Kohno, H., Shida, T., Kimura, F., Shimizu, H., Ohtsuka, M., Nakatani, Y., and Miyazaki, M. (2009). Significance of monocyte chemoattractant protein-1 in angiogenesis and survival in colorectal liver metastases. Int. J. Oncol. *34*, 923–930.

Yu, H., Pardoll, D., and Jove, R. (2009). STATs in cancer inflammation and immunity: a leading role for STAT3. Nat. Rev. Cancer *9*, 798–809.

Zhang, J., Patel, L., and Pienta, K.J. (2010). CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. Cytokine Growth Factor Rev. *21*, 41–48.

Zijlmans, H.J., Fleuren, G.J., Baelde, H.J., Eilers, P.H., Kenter, G.G., and Gorter, A. (2006). The absence of CCL2 expression in cervical carcinoma is associated with increased survival and loss of heterozygosity at 17q11.2. J. Pathol. *208*, 507–517.

Cancer Cell, Volume 22

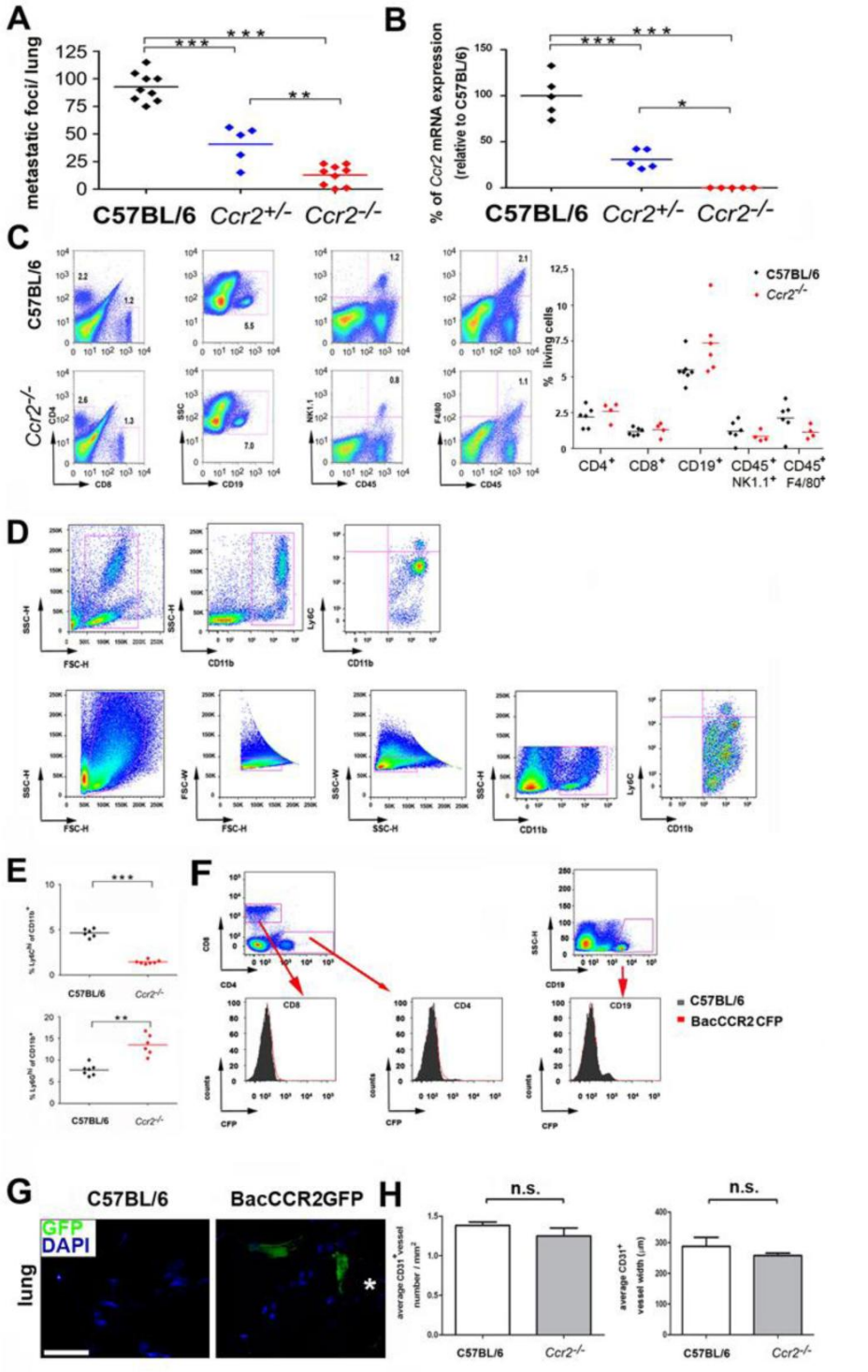
Supplemental Information

Endothelial CCR2 Signaling Induced by Colon Carcinoma Cells Enables Extravasation via the JAK2-Stat5 and p38MAPK Pathway

Monika J. Wolf, Alexandra Hoos, Judith Bauer, Steffen Boettcher, Markus Knust, Achim Weber, Nicole Simonavicius, Christoph Schneider, Matthias Lang, Michael Stürzl, Roland S. Croner, Andreas Konrad, Markus G. Manz, Holger Moch, Adriano Aguzzi, Geert van Loo, Manolis Pasparakis, Marco Prinz, Lubor Borsig and Mathias Heikenwalder

Inventory of Supplemental Information

Supplemental Data



2

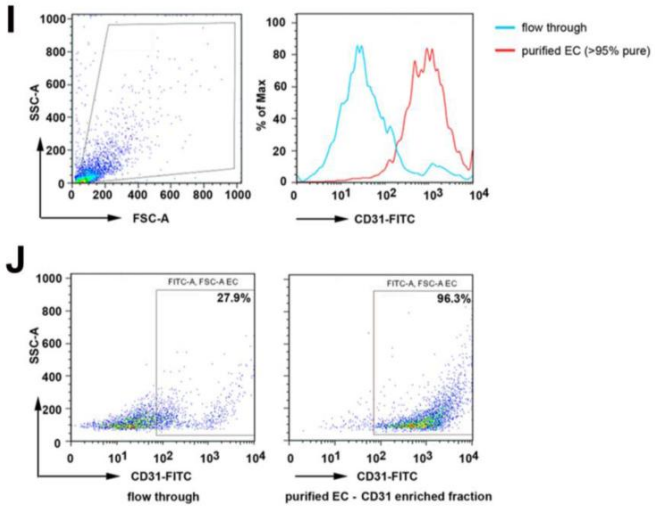


Figure S1. Related to Figure 1. Characterization of *Ccr2*$^{-/-}$ lungs in health and disease. **(A)** Quantification of tumor nodules in C57BL/6 (black), *Ccr2*$^{+/-}$ (blue) and *Ccr2*$^{-/-}$ (red) lungs on day 28 p.i. with MC-38GFP tumor cells. **(B)** Real-time PCR for *Ccr2 mRNA* expression in lung tissue derived from C57BL/6, *Ccr2*$^{+/-}$ and *Ccr2*$^{-/-}$ mice (n=5, each). Values are normalized to the average of *Ccr2 mRNA* expression in C57BL/6 lungs. **(C)** Representative flow cytometry analysis from C57BL/6 (upper row) and *Ccr2*$^{-/-}$ lungs (lower row). No significant differences were found in the numbers of CD4^{+} and CD8^{+} T-cells, CD19^{+} B-cells, NK1.1^{+} NK-cells, and F4/80^{+} macrophages in naïve lungs of C57BL/6 (black; n=5-6) and *Ccr2*$^{-/-}$ mice (red; n = 4-6). **(D)** Gating strategy used for the analysis of CD11b^{+}Ly6C^{hi} cells in peripheral blood (upper panel) and lung tissue (lower panel). **(E)** Quantification of flow cytometry analysis of CD11b^{+}Ly6C^{hi} monocytes (upper panel) and CD11b^{+}Ly6G^{+} cells (lower panel) in naïve lungs of C57BL/6 (black) and *Ccr2*$^{-/-}$ mice (red) (n = 6-7, each). **(F)** Flow cytometry analysis using BacCCR2CFP mice for the presence of CFP (indicative of an activate CCR2 promoter) on CD8^{+} and CD4^{+} T-cells as well as CD19^{+} B-cells. Histograms reveal no CFP expression on T-cells and on B-cells in BacCCR2CFP mice (red line) compared to C57BL/6 (gray, filled) mice. **(G)** Confocal microscopy image of immunofluorescent staining for GFP expression (green) on lung tissue sections of

BacCCR2GFP and C57BL/6 mice for control. Asterisk indicates the alveolar space. **(H)** Densitometric analysis of the average number of $CD31^+$ vessels/mm^2 and the average width of $CD31^+$ vessels (mean +/- SEM) in lung tissue of C57BL/6 and *Ccr2*$^{-/-}$ mice. Statistical significance: ***p < 0.001; **p < 0.01; *p < 0.05; n.s. = not significant. **(I)** Flow cytometry analysis to control for the purity of CD31-sorted pulmonary endothelial cells from C57BL/6 or *Ccr2*$^{-/-}$ mice. Left panel: Forward scatter (FSC) and sideward scatter (SSC) of a representative flow cytometry plot for cells analyzed from a lung preparation. Right panel: Histogram-analysis for CD31 protein expression of the sorted $CD31^+$ fraction derived from a representative lung preparation as well as the flow through fraction for control. EC: endothelial cells. **(J)** Representative flow-through and CD31-sorted fractions analyzed by flow cytometry. Percent of CD31-expressing cells are indicated. A purity of around 95% was reached making it possible to cultivate and analyze endothelial cells in the subsequent experiments.

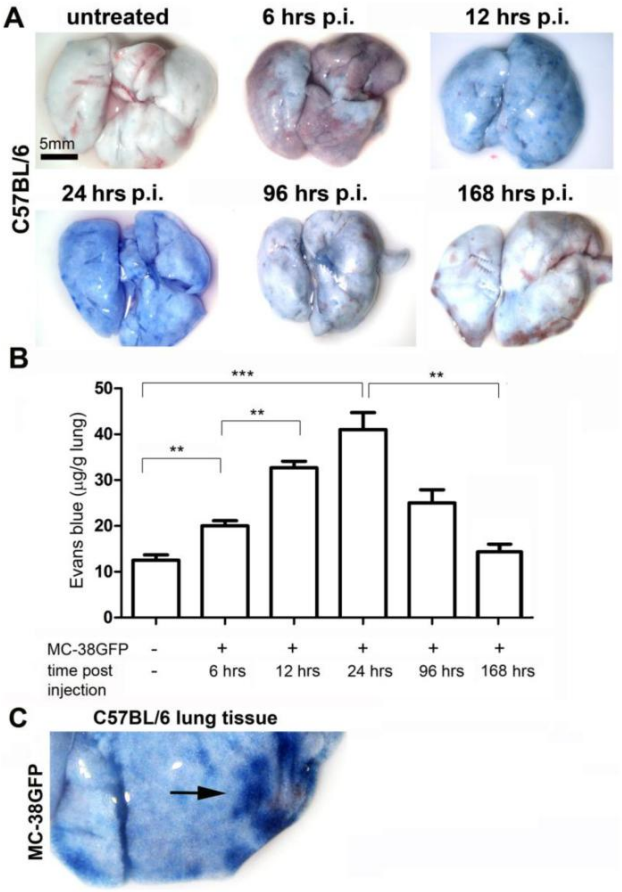


Figure S2. Related to Figure 2. Time course to study lung permeability upon tumor cell challenge. (A) Macroscopy of lungs derived from C57BL/6 mice to study the kinetics of Evans blue accumulation in lung after injection of MC-38GFP tumor cells. Various time points between 6 hrs and 7 days post challenge with MC-38GFP are shown. Size of scale bar is indicated. **(B)** Quantification of Evans blue by spectrophotometric analysis (mean +/- SEM) in lung homogenates of C57BL/6 mice at 6 hrs, 12 hrs, 24 hrs, 96 hrs and 168 hrs p.i. with MC-38GFP, showing that the induction of lung permeability is transient and a naïve state is reached at 168 hrs p.i. (n = 3-5, for each time point). Statistical significance: ***$p < 0.001$; **$p < 0.01$. **(C)** High magnification of Evans blue accumulation in lung tissue of a C57BL/6 mouse 24 hrs p.i. with MC-38 cells showing focal Evans blue staining.

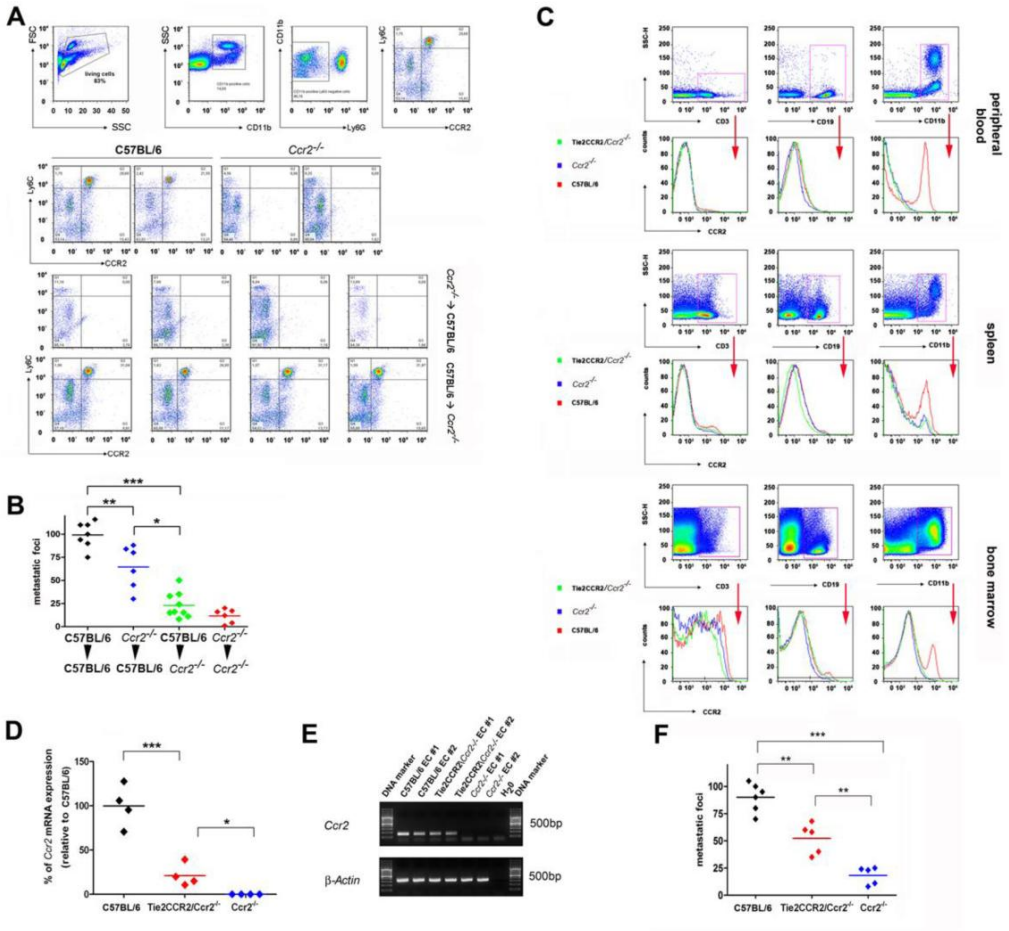


Figure S3. Related to Figure 3. Reconstitution efficacy of chimeric mice and characterization of Tie2CCR2/*Ccr2*$^{-/-}$ mice. (A, upper row) Gating strategy used for the analysis of CCR2 expression on Ly6C^{hi} cells in blood. **(A, lower rows)** Representative flow cytometry analysis of control and chimeric mice. **(B)** Quantification of metastatic foci in lungs of chimeric mice 28 d.p.i. with MC-38GFP tumor cells. C57BL/6→C57BL/6 (black; n = 8), *Ccr2*$^{-/-}$→C57BL/6 (blue; n = 6); C57BL/6→*Ccr2*$^{-/-}$ (green; n = 9) and *Ccr2*$^{-/-}$→*Ccr2*$^{-/-}$ (red; n = 6) mice are depicted. **(C)** Flow cytometry analysis for the expression of CCR2 on CD3^{+} T-cells, CD19^{+} B-cells and CD11b^{+} monocytes in the blood (upper panel), spleen (middle panel) and BM (lower panel) of C57BL/6 (red), Tie2CCR2/*Ccr2*$^{-/-}$ (green) and *Ccr2*$^{-/-}$ mice

(blue). Histograms reveal no difference in the CCR2 expression pattern between Tie2CCR2/*Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice, whereas C57BL/6 mice show CCR2 expression, mainly on CD11b^{+} cells. **(D)** Real-time PCR for the expression of *Ccr2 mRNA* in lung tissue derived of C57BL/6, Tie2CCR2/*Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice. **(E)** RT-PCR for the expression of *Ccr2* in CD31-sorted endothelial cells isolated from lungs of C57BL/6, Tie2CCR2/*Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice. Two samples per genotype are shown, *β-actin* served as control (bp = base pairs). **(F)** Quantification of metastatic foci in lungs of C57BL/6, Tie2CCR2/*Ccr2*$^{-/-}$ and *Ccr2*$^{-/-}$ mice 28 d.p.i. with MC-38GFP. Statistical significance: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

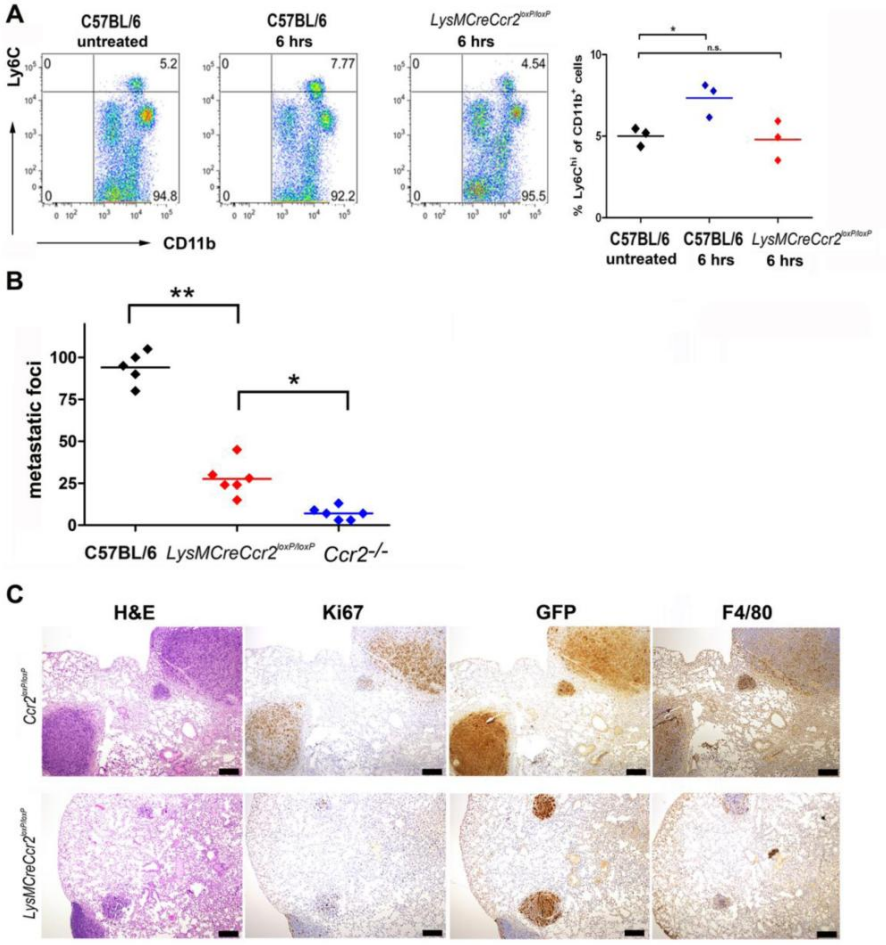


Figure S4. Related to Figure 4. Reduced but not ablated tumor growth in lungs of *LysMCreCcr2*$^{loxP/loxP}$ mice. (A) Flow cytometry analysis for CD11b^{+}Ly6C^{hi} cells in lungs of naïve C57BL/6 and MC-38-injected C57BL/6 and *LysMCreCcr2*$^{loxP/loxP}$ mice 6 hrs p.i. (n = 3; left panel). Quantification of Ly6C^{hi} cells (right panel). **(B)** Quantification of metastatic foci in lungs of C57BL/6, *LysMCreCcr2*$^{loxP/loxP}$ and *Ccr2*$^{-/-}$ mice 28 d.p.i. with MC-38GFP. Statistical significance: **p < 0.01; *p < 0.05; n.s. = not significant. **(C)** Histological analysis of MC-38GFP tumors in *Ccr2*$^{loxP/loxP}$ (upper row) and *LysMCreCcr2*$^{loxP/loxP}$ (lower row) lungs at 28 d.p.i. H&E: Hematoxylin/Eosin, Ki67 stains for proliferating cells, GFP for tumor cells, F4/80 for monocytes and macrophages. Scale bar: 200µm.

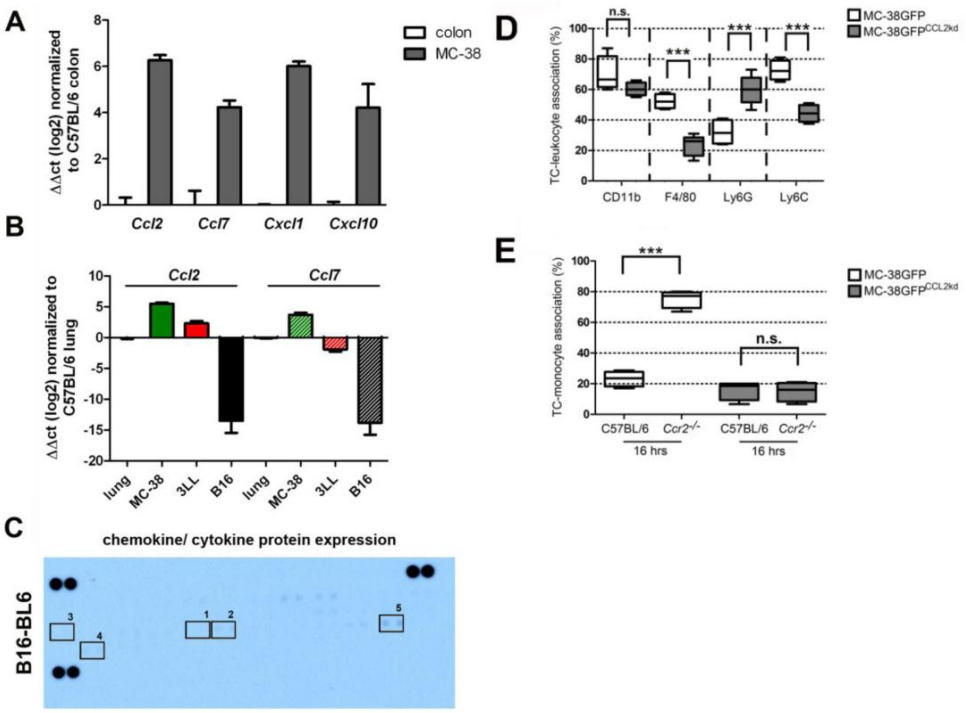


Figure S5. Related to Figure 5. Characterization of chemokine expression in various syngeneic tumor cell lines. (A) Real-time PCR analysis for *mRNA* expression of selected chemokines in MC-38 cells compared to colon samples derived from C57BL/6 mice. Data are presented as ΔΔct values on a log2 scale. MC-38 cells express high levels of *Ccl2*, *Ccl7*, *Cxcl1* and *Cxcl10* compared to *mRNA* isolated from colon of C57BL/6 mice. Each bar reflects the median expression (mean +/- SEM) of a particular gene resulting from three to four biological samples with three to four technical replicates. **(B)** Quantitative *mRNA* analysis for the expression of *Ccl2* and *Ccl7* in MC-38, 3LL and B16-BL6 compared to *mRNA* isolated from lungs of C57BL/6 mice. Data are presented as ΔΔct values on a log2 scale. Each bar reflects the median expression (mean +/- SEM) of a particular gene resulting from three to four cell line samples and three to four technical replicates, normalized to the mean expression value of the respective gene in C57BL/6 lungs. **(C)** Expression profile of various chemokines and cytokines in B16-BL6 cells. Dots in the upper right and left corners serve as loading controls. Some chemokines are marked (e.g. **1**: CCL2; **2**: CCL12; **3**: CXCL10; **4**:

TIMP-1; **5**: CCL5). **(D)** Analysis of confocal images of tumor cell interaction with endogenous myeloid cells ($CD11b^+$, $F4/80^+$, $Ly6G^+$ and $Ly6C^+$) in lungs of C57BL/6 mice challenged with MC-38GFP and MC-38GFP^{CCL2kd} 24 hrs p.i (n = 4). Percentiles are indicated (mean with min/max is shown). **(E)** Analysis of confocal images of the interaction of adoptively transferred, PKH26-labeled monocytes (injected 6 hrs post tumor cell injection) with MC-38GFP and MC-38GFP^{CCL2kd} cells in C57BL/6 and *Ccr2*$^{-/-}$ mice 16 hrs p.i (n = 4). Percentiles are shown (mean with min/max is shown); statistical significance: ***p < 0.001; n.s. = not significant.

10

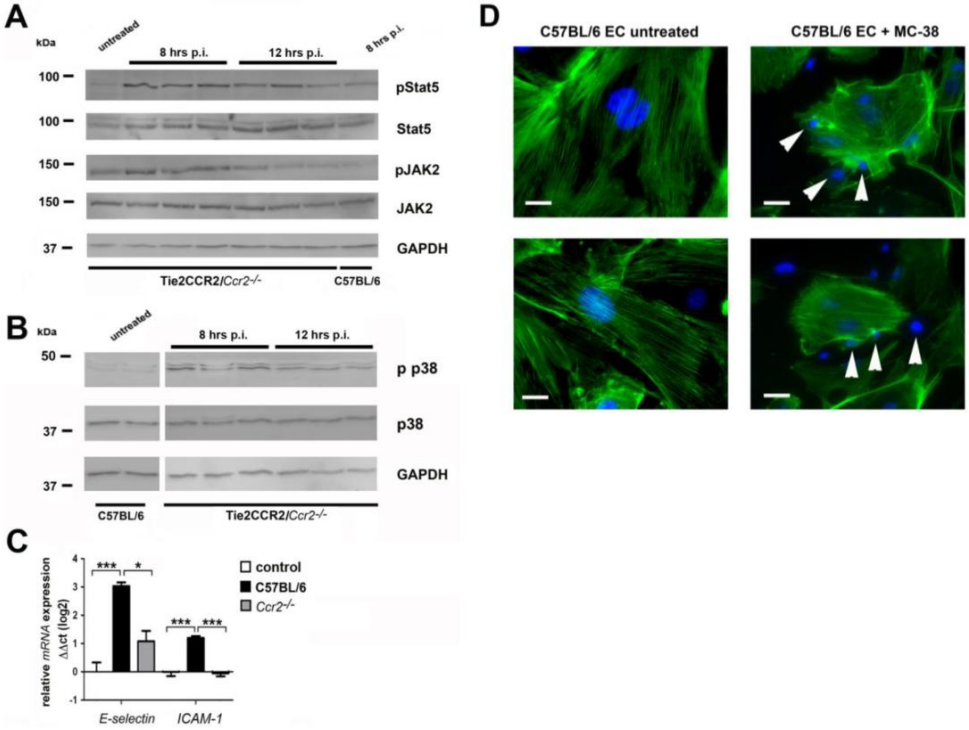


Figure S6. Related to Figure 7. Stat5 and p38MAPK activation in endothelial cells occurs in the absence of Ly6C^{hi} monocytes. (A) Immunoblot analysis for pStat5, Stat5, pJAK2, JAK2 and GAPDH in Tie2CCR2/*Ccr2*$^{-/-}$ and C57BL/6 mice, either untreated or challenged with MC-38GFP and analyzed at 8 and 12 hrs p.i. **(B)** Immunoblot analysis for p p38MAPK, p38MAPK and GAPDH in Tie2CCR2/*Ccr2*$^{-/-}$ and C57BL/6 mice, either untreated or challenged with MC-38GFP and analyzed at 8 and 12 hrs p.i. **(C)** Real time PCR analysis for the expression of *E-selectin* and *Icam-1 mRNA* in lungs of C57BL/6 (black bar) and *Ccr2*$^{-/-}$ mice (gray bar) 8 hrs p.i. with MC-38GFP (n = 3). Data are presented as ΔΔct values (log2 scale). Each bar represents the median expression (mean +/- SEM) of a gene resulting from three to four technical replicates, normalized to the mean expression value of the respective gene in C57BL/6 lungs (control). Statistics: ***p < 0.001; *p < 0.05. Furthermore, *Vcam*, *Vegfα*, *Vegfβ*, *Occludin*, *Claudin-5, Endoglin, Thrombospondin* and *VE-cadherin mRNA* levels in C57BL/6 and *Ccr2*$^{-/-}$ lungs 8 hrs p.i. with tumor cells were also investigated but

showed no altered expression in the different genotypes. **(D)** Retraction of the cytoskeleton in endothelial cells upon co-culture with MC-38 cells. Phalloidin-FITC staining of primary C57BL/6 endothelial cells left untreated (left panel) and upon co-culture with MC-38GFP cells for 12 hrs (right panel). Arrows indicate MC-38GFP tumor cells. Size of scale bar: 20μm.

Supplemental Experimental Procedures

Cell culture: Mouse colon carcinoma cell line MC-38 and MC-38 stably expressing GFP (MC-38GFP) as well as B16-BL6 melanoma cells were grown in DMEM medium with 10% FCS (Borsig et al., 2002). Lewis lung carcinoma cells (3LL) were grown in RPMI medium with 10% FCS (Läubli and Borsig, 2010).

Bone marrow reconstitutions: C57BL/6 and *Ccr2*$^{-/-}$ mice were used to generate BM chimeras. Recipient animals were irradiated with 900 rad in one dose and reconstituted by i.v. injection of purified BM cells (isolated from femur and tibia). Reconstitution efficiency was assessed by flow cytometry analysis for CCR2^{+}Ly6C^{hi} cells in the blood 6-7 weeks after reconstitution and before injection of tumor cells.

Resection of colon tumor tissue: Tumors with a distance of at least 16cm proximal the anal verge measured with a rigid endoscope were classified as colon carcinomas. Cases arising from a history of inflammatory bowel disease or hereditary syndromes (e.g. familial adenomatous polyposis) were not included. Uninvolved colon tissues (n=4) at least 10cm distant from the tumor site were harvested as controls. The patients underwent colon resection with a standardized regional lymph node dissection. This technique and standard oncological colon resections performed in our institution are described elsewhere (Hohenberger et al., 2003). Adjuvant or palliative treatment was carried out following the current German guidelines for colorectal carcinomas (Schmiegel et al., 2010).

Histology and electron microscopy: Sections (2µm) of lungs (fixed in 4% paraformaldehyde and paraffin-embedded) were stained with Hematoxylin/Eosin or various antibodies. Incubation in Ventana buffer and staining was performed on a NEXES immuno-histochemistry robot (Ventana Instruments) using an IVIEW DAB Detection Kit (Ventana) or on a Bond MAX (Leica). Antibodies against murine B220^{+} B-cells (Pharmingen; 1:400), F4/80 (Serotec, 1:50) for macrophages, CD3^{+} T-cells (clone SP7, Neomarkers; 1:300) were kindly provided by R. Zinkernagel (Odermatt et al., 1991). Anti-GFP antibody (Fitzgerald Industries

International, 1:1000) was used to visualize MC-38GFP cells. Ki67 (NeoMarkers; 1:200) stained proliferating tumor cells and lymphocytes. Image acquisition was performed on an Olympus BX53, equipped with an Olympus DP72 camera using cellSens software or on an Olympus SZX12, equipped with a JVC digital camera (KY-F70; 3CCD) using Analysis software.

For electron microscopy, sections from epon-embedded, glutaraldehyde-fixed lungs were cut and stained with toluidine blue. The tissue was trimmed and ultrathin cross sections of the lung were cut and treated with uranyl acetate and lead citrate as described previously (Raasch et al., 2011). Electron micrographs were analyzed for cell composition and localization using the analySIS Docu System (Soft Imaging System GmbH).

Experimental metastasis assay: For experiments with 3LL and B16-BL6 cells, $1.5x10^5$ cells were i.v. injected and numbers of tumor nodules were counted on day 12 or day 14, respectively. For co-injection experiments with GFP positive and negative tumors, lungs were fixed in 4% paraformaldehyde and embedded in paraffin blocks. Paraffin blocks were then analysed by series of step cuts. Each 300μm, 4 sections were cut and subsequently stained for the respective markers (e.g. GFP, H&E, Ki67). Lung tissue was cut through and 3-5 different levels (all separated by 300μm) were analysed per lung.

Endothelial cell staining: Lungs were intratracheally injected with agarose and 100μm sections cut on a vibrating microtome (Leica VT1200). Sections were incubated with 5μg/ml anti-CD31 antibody (BD Biosciences) followed by 2μg/ml Alexa594-conjugated anti-rat IgG (Molecular Probes). Nuclei were counterstained with 14nM DAPI. Fluorescent images were collected sequentially in three channels on a TCS-SP5 confocal microscope (Leica Microsystems). Series of optical sections of 5 to 15μm were collected and maximum projections are shown. Images were exported from the Leica software and processed with Adobe Photoshop CS3.

For densitometric analysis of $CD31^+$ vessels, sections were stained with 1μg/ml anti-CD31 antibody followed by AP-conjugated anti-rat IgG on a Leica BONDMAX. At least three

images (2.2mm x 1.7mm) of each mouse (C57BL/6: n=2; *Ccr2*$^{-/-}$: n=3) were taken and CD31^{+} vessels were quantified.

Tumor cell survival: Cryosections of lungs were prepared at different time points after i.v. injection of MC-38GFP cells as described previously (Borsig et al., 2001). Lung sections were stained with DAPI and the number of surviving GFP^{+} tumor cells was counted per view field at 40x magnification.

Tumor cell extravasation assay: Mice were i.v. injected with MC-38GFP cells and after 24 hrs lungs were perfused with PBS followed by perfusion with 100μg tomato lectin conjugated with Texas red (Vector Laboratories). After 10 mins, lungs were again perfused with PBS, fixed and frozen as described previously (Borsig et al., 2001). Frozen lung sections (8μm) were stained with DAPI. Three-dimensional images were captured with a SP5 confocal microscope (Leica) of total 5μm in a z-section and further analyzed with Imaris software (Bitplane). The contact area of tumor cells with the lectin-stained vasculature was determined.

Analysis of leukocyte-tumor cell association: Cryosections (8μm) prepared from lungs of mice i.v. injected with MC-38GFP cells were stained with following antibodies: CD11b for myeloid cells, Ly6G for neutrophils, biotinylated Ly6C for inflammatory monocytes (all BD Biosciences) and F4/80 (AbD Serotec) for macrophages. Alexa568-conjugated anti-rat IgG or Streptavidin-Alexa568 (Invitrogen) were used for the visualization of signals with a fluorescence microscope (Zeiss). Tumor cells were counted and the percentage of tumor cells associated with leukocytes was determined.

Adoptive transfer: Monocytes derived from C57BL/6 mice were prepared by conditional derivation of primary BM progenitors as described previously (Sykes and Kamps, 2001). Differentiated monocytes were characterized by expression of F4/80, CD11b, and Ly6C staining (L. Borsig, unpublished observations). *In vitro* differentiated monocytes were labeled with PKH26-Red Fluorescent Cell Linker (Sigma), and 2x10^{5} labeled cells were infused i.v. 6

hrs p.i. of MC-38GFP. After 16 hrs, mice were terminated, lungs were perfused with PBS and frozen as previously described (Borsig et al., 2001). Frozen lung sections (8μm) were stained with DAPI. The extent of tumor cell association with macrophages was visualized with a SP5 confocal microscope (Leica) and quantified.

RNA isolation from mouse tissue: Total RNA from flash frozen, PBS-perfused lungs, colon from C57BL/6 mice and cell pellets was isolated using RNeasy Mini Kit (Qiagen). The quantity and quality of the RNA was determined spectroscopically using a Nanodrop (Thermo Scientific).

RNA isolation from human colon tumor tissue: RNA was isolated using a fully automated extraction method from FFPE tissue (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics) has been described previously (Hennig et al., 2010). In brief, 5μm paraffin sections were directly subjected to automated total nucleic acid extraction. Samples were heat-lysed in 150μL FFPE buffer at 80ºC for 30 mins with shaking. After cooling, enzymatic lysis was carried out at 65ºC for 30 mins with proteinase K. Any residual tissue debris was removed by nonspecific binding to silica-coated iron-oxide beads and subsequent magnetic separation. Deparaffinized and clarified lysates were transferred to new tubes and nucleic acids were bound to fresh silica-coated beads under chaotrophic conditions. Beads were washed 3 times and total nucleic acids were eluted with 100μL of elution buffer at 70ºC followed by an automated DNase I digestion.

Real-time PCR: Purified RNA was reversely transcribed into cDNA using Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. For *mRNA* expression analysis real-time PCR was performed using Fast Start SYBR Green Master Rox (Roche). Primers were custom made by Microsynth. Real-time PCR was performed on an ABI PRISM 7900 HT Fast Real-Time PCR System (AB). Data were generated and analyzed using SDS 2.4 and RQ manager 1.2 software. For murine samples, *mRNA* expression levels were normalized to the housekeeping gene *Gapdh*, primer sequences used are listed below. For RT-PCR, *β-actin* served as a housekeeping gene and samples were run on a 2% agarose

gel and images were acquired using red gel imaging system from Alpha Innotech with Pronto software. For human samples, *GAPDH, RPL37a* and *β-ACTIN* served as housekeeping genes and *CCL2* expression levels were determined using the following primers: CCL2-FWD: 5'-GAA GCT CGC ACT CTC GCC TCC-3' and CCL2-REV: 5'-TGA GCG AGC CCT TGG GGA ATG A-3'

gene	fwd (5'-3') primer	rev (5'-3') primer
Ccl2	TTA AAA AAC CTG GAT CGG AAC CAA	GCA TTA GCT TCA GAT TTA CGG GT
Ccl3	TTC TCT GTA CCA TGA CAC TCT GC	CGT GGA ATC TTC CGG CTG TAG
Ccl4	TTC CTG CTG TTT CTC TTA CAC CT	CTG TCT GCC TCT TTT GGT CAG
Ccl5	GCT GCT TTG CCT ACC TCT CC	TCG AGT GAC AAA CAC GAC TGC
Ccl7	GCT GCT TTC AGC ATC CAA TGT	CCA GGG ACA CCG ACT ACT G
Ccl8	TCT ACG CAG TGC TTC TTT GCC	AAG GGG GAT CTT CAG CTT TAG TA
Ccl12	ATT TCC ACA CTT CTA TGC CTC CT	ATC CAG TAT GGT CCT GAA GAT CA
Cx3cl1	GGG TGG CCA TGT TTG CTT AC	CAG GCA AGC AGC TCA CAC TG
Cxcl1	TTT GTC ACC AAA CGA GGA CTA AA	CCA GTC AGG GTT ATC GCT GTG
Cxcl10	AAG TGC TGC CGT CAT TTT CT	CCT ATG GCC CTC ATT CTC AC
Ccr1	TTT TAA GGC CCA GTG GGA GTT CAC TCA CGG	TGG TAT AGC CAC ATG CCT TTG AAA CAG CTG C
Ccr2	CTA CGA TGA TGG TGA GCC TTG	GAT ATC AGT CAT GCT CTT CAG C
Ccr4	GGA AGG TAT CAA GGC ATT TGG G	GTA CAC GTC CGT CAT GGA CTT
Ccr5	TTT TCA AGG GTC AGT TCC GAC	GGA AGA CCA TCA TGT TAC CCA C
Ccr7	TGT ACG AGT CGG TGT GCT TC	GGT AGG TAT CCG TCA TGG TCT TG
Ccr8	ACG TCA CGA TGA CCG ACT ACT	CCC AGC ACA AAC AAG ACG C
Cxcr1	TCT GGA CTA ATC CTG AGG GTG	GCC TGT TGG TTA TTG GAA CTC TC
Cxcr2	GTG CTC CGG TTG TAT AAG ATG AC	ATG CCC TCT ATT CTG CCA GAT
Cxcr4	GCT GGC TGA AAA GGC AGT CTA T	TGA CGT CGG CAA AGA TGA AGT
Cx3cr1	GTC TTC ACG TTC GGT CTG GTG	TGG CTG ATG AGG TAG TGA GTC
E-selectin	ATG CCT CGC GCT TTC TCT C	GTA GTC CCG CTG ACA GTA TGC
I-cam1	GCT ACC ATC ACC GTG TAT TCG	TAG CCA GCA CCG TGA ATG TG
Gapdh	CCA CCC CAG CAA GGA GAC	GAA ATT GTG AGG GAG ATG CT
β-actin	GAC GGC CAG GTC ATC ACT AT	ACA TCT GCT GGA AGG TGG AC

Flow cytometry analysis: Blood and lung cell samples were prepared at 4°C in buffer solution (PBS containing 2% FCS and 2mM EDTA) and stained against CD45, CD19, CD4, CD8, Ly6G, NK1.1 (all BD Biosciences), Ly6C, F4/80 (eBioscience), and/or CCR2 (Mack et al., 2001). For flow cytometry analysis of mononuclear cells in lung tissue, mice were perfused with PBS and lungs were removed, minced and digested under shaking conditions for 1 hr at 37°C in 2mg/ml Collagenase D (Roche). Cells were separated using 40µm cell strainers. For the analysis of blood samples, erythrocytes were lysed with BD FACS lysing

solution and cell suspensions were analyzed on a FACS Calibur and FACS Canto II (BD Biosciences). Data were acquired using Cell Quest software 6.0 (FACS Calibur) and Facs Diva software v6.1.3 (Facs Canto II) analyzed using Flow Jo (v7.6 for FACS Calibur data and v8.8.4 for FACS Canto II data). For the analysis of spleen and BM samples, spleens were minced and BM cells were isolated from tibia and femur and stained as described above.

Cytokine protein array: For cytokine expression pattern of cell lines, Proteome Profiler arrays (mouse cytokine array panel A; R&D Systems) were used according to the manufacturers protocol. In brief, flash frozen cell pellets were lysed in buffer containing 20mM Tris HCl pH 8.0, 137mM NaCl, 2mM EDTA and 1% NP40. Protein concentration was determined using BCA protein assay reagent (Thermo Scientific) and 50μg protein was used for each protein array. Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) on Min-R 2000 films (Kodak) and quantified using Aida software (Raytest).

Immunoblot analysis: Lung homogenates (10%) were prepared in RIPA buffer (50mM Tris; 1% NP40; 0.25% Deoxycholic acid sodium salt; 150mM NaCl; 1mM EGTA) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), and quantified with a BCA protein assay kit (Thermo Scientific) according to the manufacturer's manual. 50μg protein were denatured in Laemmli buffer containing 5% β-mercaptoethanol and separated by gel electrophoresis on 8% or 11% Tris-glycine SDS-PAGE with Tris-glycine running buffer and blotted by semi-dry blotting (Trans-Blot SD cell, Biorad) onto a nitrocellulose membrane (Protran BA 85 pore size 0.45μm; Whatman). After blotting, the membrane was blocked in 5% BSA-TBST for 2 hrs at RT. Primary antibodies against pJAK2, JAK2, pStat5, Stat5, P-p38MAPK and p38MAPK (all Cell Signaling, 1:1000 dilution) were incubated at 4°C over night under shaking conditions. Incubation with the secondary antibody (HRP-anti rabbit IgG, 1:5000; Promega) was performed under shaking conditions for 1 hr. Detection was achieved with Supersignal West chemiluminescent substrate (Pierce) using Stella 3200 imaging

system (Raytech). To assure equal loading, the membranes were reprobed with anti-GAPDH antibody (Chemicon) and detected as described above.

Isolation of bone marrow monocytes: Long bones from 6-8 week old mice were flushed with PBS containing 2% FCS and 2.5mM EDTA. Red blood cells were lysed using ammonium chloride solution. BM nucleated cells were pre-enriched by magnetic activated cell sorting (MACS) using biotinylated M-CSFR antibody (Biolegend) and streptavidin-conjugated magnetic beads (Miltenyi Biotec). Following staining with Gr-1 (Ly6G) monoclonal antibody conjugated to phycoerythrin and streptavidin-allophycocyanin (eBioscience), BM monocytes were sorted as M-CSFR^{+}Gr-1^{int} cells using a FACSAria III cell sorter (BD Biosciences).

Generation of knock-down cells: Silencing of CCL2 in tumor cells was achieved by viral transduction with shRNA lentivirus construct (Sigma NM_002982). Tumor cells were seeded on 24-well plates ($3x10^4$) until 50% confluence and incubated with lentivirus for 16 hrs. Cells were passaged after 48 hrs and plated into 6-well plates in the presence of puromycin (10μg/ml). Three days later, cells were split again and the knock-down efficiency was determined by quantitative PCR and immunoblotting. Lentivirus containing a scrambled shRNA construct was used for preparation of control cells.

Isolation of pulmonary endothelial cells: Pulmonary endothelial cells were isolated using a positive immuno-magnetic selection as described previously (Wang et al., 2005). Briefly, perfused lungs were removed, minced and digested in 0.1% collagenase A (Roche). A single-cell suspension was generated by passing the digested lungs through 100μm and 40μm cell strainers (BD Falcon). Cells were incubated with FITC-labeled anti-CD31 antibody (Invitrogen) for 30 mins at 4°C. Anti-rat IgG beads (Miltenyi Biotec) were added to the cells and incubated for 15 mins at 4°C, followed by magnetic separation. The purity of isolated endothelial cells was determined by flow cytometry and isolated endothelial cells were cultured in gelatin-coated 6-well plates in medium containing endothelial cell growth supplement (BD Biosciences).

Transendothelial migration assay: Primary lung microvascular endothelial cells ($3x10^4$) were seeded on gelatin coated 24-well transwell inserts (8μm pores; BD Biosciences) and allowed to grow to confluence for 2 days. Tumor cells ($2x10^4$) were seeded into transwell inserts with or without monocytes ($1x10^5$) in 5% FCS/RPMI in the upper chamber and 10% FCS/RPMI in the lower chamber. After 16 hrs of co-culture, the upper side of the insert was scraped off and the insert was fixed in 1.5% paraformaldehyde. The transwell membrane was removed and mounted on a slide. Tumor cells on the lower side of the membrane as well as in the lower wells were analyzed with a fluorescence microscope (Zeiss). Monocytes were labeled with DiL (Molecular Probes) and transmigrated cells counted in the lower wells. For inhibitor studies, AG490 (8μM final concentration), NSC23766 (20μM), SB202190 (10μM), S3I-201 (50μM), Wortmannin (1μM) and Stat5 inhibitor (100μM) were added to the culture media in the upper and the lower chamber of the 24-well plates. For the permeability assay the inserts were prepared as mentioned above. MC-38 and MC-38^{CCL2KD} cells were incubated with or without monocytes for 7 hrs. Transwell inserts were transferred into the new plate containing PBS. The medium in the upper chamber was replaced with FITC-Dextran (0.5g/ml) and incubated for 15 mins. Fluorescence was measured in 100μl of the sample from the lower chamber. All experiments were performed in duplicates and repeated several times.

Staining of endothelial cytoskeleton: To demonstrate endothelial response to tumor cells, the cytoskeleton was stained with Phalloidin (Zarbock et al., 2006). Briefly, $2x10^4$ pulmonary endothelial cells were grown on gelatin-coated chamber slides for 48 hrs, until ~80% confluency was achieved. MC-38GFP cells ($1x10^4$) were added to the endothelial monolayers. After 12 hrs cells were fixed with 1.5% paraformaldehyde, permeabilized (0.1% saponin), and stained with Phalloidin-FITC (40mg/ml, Invitrogen). Nuclei were stained with DAPI and mounted in ProLong Gold (Invitrogen). Slides were analyzed with an Axiovert 200M (Zeiss) microscope.

Statistical analysis: Statistical analysis was performed with the GraphPad Prism software (version 4.0). All data are presented as mean ± SEM and were analyzed by ANOVA with the post-hoc Bonferroni multiple comparison test, unless specified differently. Analysis of two samples was performed with Student *t* test.

Supplemental References

Borsig, L., Wong, R., Feramisco, J., Nadeau, D. R., Varki, N. M., and Varki, A. (2001). Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci USA *98*, 3352-3357.

Borsig, L., Wong, R., Hynes, R. O., Varki, N. M., and Varki, A. (2002). Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc Natl Acad Sci USA *99*, 2193-2198.

Hennig, G., Gehrmann, M., Stropp, U., Brauch, H., Fritz, P., Eichelbaum, M., Schwab, M., and Schroth, W. (2010). Automated extraction of DNA and RNA from a single formalin-fixed paraffin-embedded tissue section for analysis of both single-nucleotide polymorphisms and mRNA expression. Clin Chem *56*, 1845-1853.

Hohenberger, W., Reingruber, B., and Merkel, S. (2003). Surgery for colon cancer. Scand J Surg *92*, 45-52.

Läubli, H., and Borsig, L. (2010). Selectins as mediators of lung metastasis. Cancer Microenviron *3*, 97-105.

Mack, M., Cihak, J., Simonis, C., Luckow, B., Proudfoot, A. E., Plachy, J., Bruhl, H., Frink, M., Anders, H. J., Vielhauer, V*., et al.* (2001). Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. J Immunol *166*, 4697-4704.

Odermatt, B., Eppler, M., Leist, T. P., Hengartner, H., and Zinkernagel, R. M. (1991). Virus-triggered acquired immunodeficiency by cytotoxic T-cell-dependent destruction of antigen-presenting cells and lymph follicle structure. Proc Natl Acad Sci USA *88*, 8252-8256.

Raasch, J., Zeller, N., van Loo, G., Merkler, D., Mildner, A., Erny, D., Knobeloch, K. P., Bethea, J. R., Waisman, A., Knust, M*., et al.* (2011). IkappaB kinase 2 determines oligodendrocyte loss by non-cell-autonomous activation of NF-kappaB in the central nervous system. Brain *134*, 1184-1198.

Schmiegel, W., Pox, C., Reinacher-Schick, A., Adler, G., Arnold, D., Fleig, W., Folsch, U. R., Fruhmorgen, P., Graeven, U., Heinemann, V*., et al.* (2010). S3 guidelines for colorectal carcinoma: results of an evidence-based consensus conference on February 6/7, 2004 and June 8/9, 2007 (for the topics IV, VI and VII). Z Gastroenterol *48*, 65-136.

Sykes, D. B., and Kamps, M. P. (2001). Estrogen-dependent E2a/Pbx1 myeloid cell lines exhibit conditional differentiation that can be arrested by other leukemic oncoproteins. Blood *98*, 2308-2318.

Wang, L., Fuster, M., Sriramarao, P., and Esko, J. D. (2005). Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. Nat Immunol *6*, 902-910.

Zarbock, A., Singbartl, K., and Ley, K. (2006). Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. J Clin Invest *116*, 3211-3219.

Manuscript 2

Selectin ligands on leukocytes are required for monocyte recruitment and adhesion at metastatic sites

Article is submitted to Journal of Cancer Research

Authors: Alexandra Hoos, Darya Protsyuk and Lubor Borsig

Contributions: I suggested, planned, conducted and analyzed all data summarized in this manuscript except the figure 5D, wrote the manuscript. D.P. provided figure 5D. L.B. suggested experiments, helped with mice work and data analysis, corrected the manuscript.

Manuscript

Selectin ligands on leukocytes are required for monocyte recruitment and adhesion at metastatic sites

Alexandra Hoos*, Darya Protsyuk, Lubor Borsig

Institute of Physiology, University of Zurich and Zurich Center for Integrative Human Physiology, CH-8057 Zurich, Switzerland

*Cancer Biology Ph.D. Program, Zurich

Corresponding author: Lubor Borsig
Institute of Physiology
University of Zurich
Winterthurerstrasse 190
CH-8057 Zurich, Switzerland
Phone: +41 44 635-5134
Fax: +41 44 635-6814
Email: lborsig@access.uzh.ch

Running Title: Selectin ligands on leukocytes facilitate metastasis

Keywords: selectin ligands, selectins, metastasis, monocytes, CCL2, neutrophils

Abstract

Tumor cell-derived selectin ligands mediate contact to the endothelium, platelets, and leukocytes through binding to selectins that facilitates metastasis. Here we describe the mechanism how endogenous (non-tumor derived) selectin ligands contribute to metastasis using Fucosyltransferase-7 (*Fuc-TVII*$^{-/-}$) deficient mice. Experimental metastasis of MC-38GFP and 3LL carcinoma cells was attenuated in *Fuc-TVII*$^{-/-}$ mice which express minimal amount of selectin ligands. We show that metastasis is dependent on selectin ligands carried on hematopoietic cells. Reduced recruitment of monocytes to metastasizing tumor cells in *Fuc-TVII*$^{-/-}$ mice correlated with attenuated metastasis. Adoptive transfer of Fuc-T7^{+} monocytes rescued metastasis in *Fuc-TVII*$^{-/-}$ mice indicating that selectin ligand-dependent recruitment of monocytes is required for successful metastasis. Cytokine analysis in metastatic lungs revealed high expression of CCL2 in C57BL/6 mice that was significantly lower in *Fuc-TVII*$^{-/-}$ mice. The absence of monocyte recruitment in *Fuc-TVII*$^{-/-}$ mice correlated with increased apoptosis of tumor cells. Thus, the recruitment of monocytes to metastasizing tumor cells is facilitated by endogenous selectin ligands carried by monocytes that enable efficient tumor cell extravasation and metastasis.

Introduction

During hematogenous metastasis circulating tumor cells interact with blood constituents (platelets and leukocytes), which modulate their capacity to adhere to endothelium and to leave blood circulation. Malignant transformation is associated with alteration of cell surface glycosylation and presentation of altered glycan structures [1; 2]. Furthermore, the enhanced expression of sialyl-Lewis^x (sLe^x) and/or sialyl-Lewis^a (sLe^a) is frequently associated with poor prognosis of carcinoma patients due to metastasis [3; 4; 5].

There is substantial evidence that selectins contribute to metastatic spread through mediation of tumor cell interaction within the circulation (reviewed in [1; 6; 7]). Selectins is a family of cell adhesion molecules, expressed on activated endothelium (E- and P-selectin), leukocytes (L-selectin) and on activated platelets (P-selectin) which are involved in different physiological situations ranging from thrombosis, inflammation, hemostasis and cancer [1; 8]. In the context of cancer progression, P-selectin facilitate platelet-tumor cell thrombi formation [9], L-selectin mediates leukocyte recruitment [10], and E-and P-selectin are required for adhesion of tumor cells to endothelium [11; 12]. The direct involvement of tumor cell-derived selectin ligands in metastasis has been shown both *in vitro* and *in vivo* [13; 14; 15]. Enzymatic removal of selectin ligands, carcinoma mucins, resulted in reduced selectin ligand interactions and subsequently attenuation of metastasis [15]. In addition, carcinoma mucins alone induced thrombosis that was selectin-dependent and required bidirectional signaling between neutrophils and platelets [16]. However, there is limited data on the involvement of endogenous (non-tumor derived) selectin ligands during cancer development and metastasis.

Selectins mediate the recruitment of leukocytes to inflammatory sites through binding to endogenous selectin ligands, expressed both on leukocytes and in the stromal

compartment [17; 18]. The mechanism of leukocyte recruitment, however, is not unique for cancer situation and has many parallels with inflammation [8; 19]. Leukocyte infiltration and secretion of cytokines contribute to metastasis through the recruitment of myeloid-derived cells that promote tumor cell extravasation [20; 21; 22]. Previously we have shown that leukocyte recruitment to metastatic sites was L-selectin-dependent [10]. Furthermore, increased L-selectin ligand expression was detected in the microenvironment of metastasizing tumor cells, but the identity of cells and the nature of the ligands remained unclear. The present study aims to elucidate the role of endogenous selectin ligands in metastasis using *Fuc-TVII*$^{-/-}$ mice that is deficient in selectin ligands.

Material and Methods

Mice: Animals were maintained under specific pathogen-free conditions, and experiments were approved and conform to the guidelines of the Swiss Animal Protection Law, Veterinary Office, Kanton Zurich. C57BL/6 mice were purchased from the Jackson Laboratory. Fucosyltransferase 7-deficient mice (*Fuc-TVII$^{-/-}$)* in a C57BL/6 background were kindly provided by Dr. J.B. Lowe (University of Michigan, Ann Arbor, MI) and bred in house.

Cell culture: Mouse colon carcinoma cell line MC-38 stably expressing GFP (MC-38GFP) was grown in DMEM medium with 10% FCS [23]. Lewis lung carcinoma cells (3LL) were grown in RPMI medium with 10% FCS [24].

Quantification of Metastasis: Mice were intravenously (i.v.) injected with MC-38GFP cells (3 x 10^5) and euthanized after 28 days. Metastatic foci were counted and macroscopic pictures of lungs were taken. 3LL (1.5 x 10^5) were i.v. injected and tumor nodules were counted on day 14.

Histology and immunohistochemistry: Lungs fixed in 4% paraformaldehyde were embedded in paraffin blocks. Lungs sections (2 µm) were stained with Hematoxylin/Eosin or various antibodies: Ki67 (Cl. SP6, NeoMarkers), anti-CD3 (Cl. SP7, NeoMarkers), anti-F4/80 (Serotec), anti-Ly6G (Becton Dickinson (BD), anti-B220 (BD). For apoptosis assay anti-Caspase-3 (Cell Signaling) and anti-GFP (Fitzgerald Industries Int.) antibodies were used. Staining was performed on a NEXES immuno-histochemistry robot (Ventana instruments, Switzerland) using an IVIEW DAB Detection Kit (Ventana) or on a Bond MAX (Leica). Images were digitalized on Zeiss Mirax Midi Slide Scanner and analyzed with Mirax Viewer 1.12. software.

Bone marrow reconstitutions: C57BL/6 and *Fuc-TVII*$^{-/-}$ mice were used for generation of bone marrow chimeras. Recipient animals were irradiated with 9 Gy in one dose and reconstituted by i.v. injection of 1x10^{7} bone marrow cells (isolated from femur and tibia). Reconstitution efficiency was analyzed by the presence of selectin ligands in the peripheral blood leukocytes and the amount Ly6G^{+} cells in the blood 6-7 weeks after reconstitution.

Flow cytometry: Mice were heart-perfused with PBS. Lungs were minced and digested with 2mg/ml Collagenase D (Roche) and 1mg/ml Collagenase A (Roche) for 1 h at 37°C. Cells were separated using 40 µm cell strainers. Red blood cells were lysed using PharmLyse (BD). Cells were incubated with rat anti-mouse CD16/32 mAb (BD) and stained with fluorophore-conjugated antibodies against: CD45, CD11b, F4/80, Ly6G, Ly6C, CD19, CD4, CD8 (BD). Anti-CD31-FITC antibody (Invitrogen) was used as an internal control for lung digestion. The number of tumor cells (MC-38GFP) in the lungs was quantified as the amount of GFP^{+} cells per 20'000 CD31^{+} endothelial cells. Blood samples were treated with PharmLyse (BD) and stained as described above. Selectin ligands were detected with mouse selectin-Fc chimeras (L-, P-, E-selectin; 10 µg/ml) that were pre-complexed with biotinylated goat-anti-human antibody (1:100; Sigma-Aldrich). Selectin binding was detected with Streptavidin-CyChrome (BD). Control samples were incubated with selectin chimera in the presence of 10 mM EDTA. Data were acquired on a BD FACSCanto II flow cytometer (BD) and analyzed using Flow Jo software (Tree Star).

Analysis of leukocyte-tumor cell association: Frozen lung sections (8-10 µm) prepared from C57BL/6 and *Fuc-TVII*$^{-/-}$ mice injected with MC-38GFP cells were stained with following antibodies: CD11b (BD), Ly6G (BD), F4/80 (AbD Serotec). Goat anti-rat-Alexa568 Ab (Invitrogen) was used to visualize signals with a

fluorescence microscope. Tumor cells were counted and the percentage of tumor cells associated with leukocytes was determined. Images were acquired with a SP2 confocal microscope (Leica) of a total 5 μm in a z-axis and analyzed with Imaris Software (Bitplane).

Real-time-PCR: Total RNA was isolated from PBS-perfused and flash frozen lungs or sorted cells (monocytes and granulocytes) from lungs using RNeasy Mini Kit (Qiagen). The quantity and quality of the RNA was determined using a Nanodrop 2000 (Thermo Scientific). Purified RNA was reversely transcribed into single stranded cDNA using Omniscript RT Kit (Qiagen) according to the manufacturer`s instructions. PCR was performed in CFX-96 thermocycler (Biorad) using a SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich) and primers specific for murine GAPDH, CCL2, CCL5, IL1β, TNFα, TGFβ. Primers purchased from Microsynth (Switzerland) are listed in the supplementary Table S1. Expression levels of target genes were normalized to the housekeeping gene *GAPDH*. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method [25].

Isolation of bone marrow monocytes: Bone marrow cell were harvested from 6-8 week old mice by flushing the long bones with PBS containing 2% FCS and 2.5 mM EDTA. Red blood cells were lysed using ammonium chloride solution. Bone marrow nucleated cells were pre-enriched using the MACS system. Briefly, cells were stained with biotinylated M-CSFR antibody (Cl. AFS98, Biolegend) and incubated with streptavidin-conjugated magnetic beads (Miltenyi Biotec). After MACS enrichment, cells were stained with Gr-1-PE mAb (Cl. RB6-8C5, eBioscience) and M-CSFR$^+$ cells were stained with streptavidin-APC (eBioscience). Bone marrow monocytes were sorted as M-CSFR$^+$Gr-1^{int} cells using a FACSAria III cell sorter (BD).

Adoptive transfer: *Fuc-TVII*$^{-/-}$ mice were intravenously injected with 2x10^5 bone marrow monocytes isolated from C57BL/6 or *Fuc-TVII*$^{-/-}$ mice, 6 h post-tumor cell injection (MC-38GFP). Mice were euthanized after 28 days and metastasis was quantified as described above.

Statistical analysis: Statistical analysis was performed with the GraphPad Prism software (version 4.0). Data are presented as mean ± SEM and were analyzed using the two-tailed Student's t-test.

Results

Endogenous selectin ligands facilitate metastasis

Selectin ligands are sialylated, fucosylated glycan structures with the core structure of sialyl Lewis^x (sLe^x). Fucosyltransferase-7 (Fuc-T7) is the major enzyme finalizing the synthesis of endogenous selectin ligands in mice [26; 27]. To assess the role of selectin ligands during metastasis, we intravenously (i.v.) injected mouse colon carcinoma cells expressing GFP (MC-38GFP) in *Fuc-TVII*$^{-/-}$ mice and lungs were analyzed 28 days later. Significant reduction in the number of metastatic foci was observed in *Fuc-TVII*$^{-/-}$ lungs compared to C57BL/6 lungs (Figure 1A-B). Immunohistological examination revealed no obvious differences in composition of Ki67$^+$ (proliferating cells), F4/80$^+$ (macrophages/monocytes) and Ly6G$^+$ (neutrophils) cells within the tumors between *Fuc-TVII*$^{-/-}$ and C57BL/6 lungs (Figure 1C). However, tumors in *Fuc-TVII*$^{-/-}$ mice showed an enhanced infiltration of B220$^+$ and CD3$^+$ cells. Of note, a higher presence of Ly6G$^+$ cells was found in lung tissues of *Fuc-TVII*$^{-/-}$ mice compared to C57BL/6 mice. The overall reduced size of metastatic lesions observed in *Fuc-TVII*$^{-/-}$ mice might be due to the accumulation of lymphocytes in tumors. However the significantly reduced numbers of metastatic nodules in *Fuc-TVII*$^{-/-}$ mice indicate the importance of endogenous (non-tumor-derived) selectin ligands during metastatic seeding.

To determine whether other tumor cells metastasize in endogenous selectin ligand-dependent manner, we injected Lewis lung carcinoma cells (3LL) into *Fuc-TVII*$^{-/-}$ mice. Similar to MC-38GFP cells, 3LL cells formed less metastatic nodules in *Fuc-TVII*$^{-/-}$ mice compared to C57BL/6 mice (Figure 1D-E). These data are consistent with attenuation of metastasis in the absence of endogenous selectin ligands. Of note, immunohistochemical analysis revealed no obvious differences in the composition of infiltrating leukocytes within the 3LL tumors compared to MC-38GFP tumors (Figure

S1). Taken together, evidence obtained from both tumor models indicates that endogenous putative selectin ligands contribute to metastatic dissemination.

We next tested whether tumor cell seeding to the lungs is dependent on endogenous selectin ligands. C57BL/6 and *Fuc-TVII*$^{-/-}$ mice were terminated at various time points after tumor cell injection and perfused lungs were analyzed for the presence of GFP^{+} tumor cells (Figure 1F-G). Similar amount of MC-38GFP cells was observed at 30 min post-injection (p.i.) in both genotypes (Figure 1G). Reduced tumor cell numbers were observed in both mice 14 h and 24 h after the injection; however a more pronounced decrease of GFP^{+} cells was detected in lungs of *Fuc-TVII*$^{-/-}$ mice. Since the initial seeding/retention of tumor cells in the lungs was comparable (30 min) and the difference in number of GFP^{+} tumor cells was detected first 14 h later, we hypothesized that endogenous selectin ligands are required for tumor cell survival and extravasation.

Selectin ligands on hematopoietic cells promote metastasis

To determine which selectin ligand-expressing cells promote metastasis, we generated bone marrow chimeric mice. Reciprocal reconstitutions (C57BL/6→*FucTVII*$^{-/-}$; *FucTVII*$^{-/-}$→C57BL/6) were performed and circulating CD11b^{+}-myeloid cells were analyzed for the presence of selectin ligands 5 weeks later (Figure 2A and S2A-B). We confirmed increased levels of Ly6G^{+} cells in the circulation of chimeric mice *FucTVII*$^{-/-}$→C57BL/6 (Figure 2B and S2) that was in line with the phenotype of *Fuc-TVII*$^{-/-}$ mice [27]. Chimeric mice were i.v. with MC-38GFP cells and the extent of metastasis was quantified after 28 days (Figure 2C-D). *Fuc-TVII*$^{-/-}$ mice expressing selectin ligands in the hematopoietic compartment (C57BL/6→*FucTVII*$^{-/-}$) displayed strong metastasis comparable to controls (C57BL/6→C57BL/6). On contrary, minimal metastasis was observed in chimeric mice expressing selectin

ligands on the radio-resistant stromal compartment (*FucTVII*$^{-/-}$→C57BL/6). These data demonstrate that selectin ligands on hematopoietic cells contribute to metastasis.

Endogenous selectin ligands facilitate monocyte recruitment

We next tested whether the absence of endogenous selectin ligands affects recruitment of leukocytes and thereby metastasis. The number of leukocytes recruited to the lungs of C57BL/6 and *Fuc-TVII*$^{-/-}$ mice injected with MC-38GFP cells was quantified 14 h and 24 h p.i. by flow cytometry (Figure 3A and S3). Interestingly, significantly higher number of leukocytes (CD45^{+} cells) was detected in lungs of untreated *Fuc-TVII*$^{-/-}$ mice compared to C57BL/6 mice. The analysis of leukocyte subpopulations revealed a 5-fold higher presence of Ly6G^{+} cells (neutrophils/granulocytes) in lungs of naïve *Fuc-TVII*$^{-/-}$ mice compared to C57BL/6 mice that was also confirmed by immunohistochemical analysis (Figure 3B). Upon tumor cell injection, increase in CD45^{+} cell infiltration was observed in both phenotypes. Of note, there was no change in number of Ly6G^{+} cells in C57BL/6 mice while further increase in *Fuc-TVII*$^{-/-}$ mice 24 h p.i. was detected. On contrary, a 3-fold increase in Ly6C^{hi} cells (inflammatory monocytes) was detected in lungs of C57BL/6 mice 14 h p.i. (Figure 3A). The numbers of Ly6C^{hi} cells in *Fuc-TVII*$^{-/-}$ mice remained on the same albeit higher level regardless of tumor cell injection. Accordingly, we observed significantly higher numbers of F4/80^{+} (macrophages) cells in C57BL/6 mice. Of note, the analysis of lymphocytes (CD8^{+}, CD4^{+} and CD19^{+} cells) revealed no changes upon tumor cell injection in both genotypes, although overall lower numbers were detected in *Fuc-TVII*$^{-/-}$ mice (Figure S3). Taken together, tumor cell-induced leukocyte infiltration to the lungs is dependent on endogenous selectin ligands, the absence of which correlated with a reduced recruitment of Ly6C^{hi} cells.

The higher numbers of Ly6G$^+$ cells observed in lungs of *Fuc-TVII*$^{-/-}$ mice seemed to be independent of selectin ligand expression.

The absence of Fuc-T7 changes myeloid cell-tumor cell interactions

To assess whether overall changes in leukocyte infiltration to the lungs alter tumor cell–myeloid cell interactions during metastatic colonization, we analyzed lung sections from C57BL/6 and *Fuc-TVII*$^{-/-}$ mice injected with MC-38GFP cells (Figure 4A). While the majority of tumor cells were associated with CD11b$^+$ cells in lungs of C57BL/6 mice at 14 h p.i., there was minimal contact observed in *Fuc-TVII*$^{-/-}$ mice. In addition, 50% of tumor cells were associated with F4/80$^+$ cells in C57BL/6 mice and the number increased at 24 h p.i. (Figure 4B). On contrary, tumor cells were minimally associated with F4/80$^+$ cells in *Fuc-TVII*$^{-/-}$ mice at any time point. Of note, despite the generally higher residency of Ly6G$^+$ cells in the lungs of *Fuc-TVII*$^{-/-}$ mice an increase in tumor cell association was observed only at 24 h p.i. (Figure 4B). Thus, the presence of endogenous selectin ligands on monocytes is required for the efficient recruitment of F4/80$^+$ cells to tumor cells during the initial phase of lung metastasis.

The reduced recruitment of leukocytes to metastasizing tumor cells could be a result of missing local expression of vascular selectins. Therefore, we tested the expression of P- and E-selectin in the tumor microenvironment in *Fuc-TVII*$^{-/-}$ mice at 6 h p.i. (Figure S4). We observed comparable expression of both P- and E-selectin in the vicinity of tumor cells in the lungs of both *Fuc-TVII*$^{-/-}$ and C57BL/6 mice indicating that the absence of endogenous selectin ligands did not interfere with the ability of tumor cells to activate the endothelium.

Monocytes carrying selectin ligands rescue metastasis in *FucTVII*$^{-/-}$ mice

Next we tested whether the injection of monocytes carrying selectin ligands affects metastasis. *Fuc-TVII*$^{-/-}$ mice were administered MC-38GFP cells that were followed by single injection of purified monocytes isolated from the bone marrow of C57BL/6 or *Fuc-TVII*$^{-/-}$ mice 6 h later. Adoptive transfer of C57BL/6 monocytes restored metastasis in *Fuc-TVII*$^{-/-}$ mice to levels comparable to C57BL/6 mice (Figure 5A-B). However, no effect on metastasis was observed after intravenous injection of selectin ligand-deficient *Fuc-TVII*$^{-/-}$ monocytes. These data demonstrate that endogenous selectin ligands on monocytes are essential for their recruitment to the lungs and therefore sufficient to promote metastasis.

Inflammatory monocytes are known to support cancer through production of cytokines, growth factors and chemokines that affect tumor cell survival, tumor growth and metastasis [19]. To check whether reduced monocyte recruitment to metastasizing tumor cells observed in *Fuc-TVII*$^{-/-}$ mice affects the microenvironment, we examined cytokines in the lungs of tumor injected mice at 24 h (Figure 5C and S5). No significant changes in expression levels of IL1β, TNF-α and CCL5 were observed after tumor cell injection in both genotypes (C57BL/6 and *Fuc-TVII*$^{-/-}$ mice). The analysis of neutrophil-specific chemokines, CXCL1, CXCL2 and CXCL5 showed minimal changes in mice of both genotypes (data not shown) indicating that the general increase in lung neutrophils observed in *Fuc-TVII*$^{-/-}$ mice is based on a different mechanism. Interestingly, the expression of CCL2 was 6-fold higher in C57BL/6 mice 14 h p.i. that remained 2-fold above the basal levels also at 24 h p.i (Figure 5C). In comparison, CCL2 expression levels were always reduced in *Fuc-TVII*$^{-/-}$ mice albeit some induction after tumor cell injection has been detected. Of note, TGFβ expression was slightly decreased in lungs of C57BL/6 mice injected with tumor cells 24 h p.i. compared to naïve lungs.

It was previously shown that CCL2 promotes tumor cell extravasation through the recruitment of monocytic cells [21; 22], and mediate a direct endothelial activation resulting in increased vascular permeability [22]. Since we observed reduced CCL2 expression levels in lungs of *Fuc-TVII*$^{-/-}$ mice we tested the lungs vascular permeability upon tumor cell injection. A comparable vascular leakiness was detected in C57BL/6 and *Fuc-TVII*$^{-/-}$ mice (Figure S6). This observation indicated that the induction of vascular permeability is independent of selectin-ligand-mediated monocyte recruitment, which is in line with previous data showing that tumor cell-derived CCL2 is mainly responsible for induction of vascular permeability [22]. Thus, lower levels of CCL2 in tumor cell-injected *Fuc-TVII*$^{-/-}$ mice might be due to reduced monocyte recruitment to the lungs. To test the hypothesis we isolated monocytes and granulocytes from tumor cell-bearing lungs and analyzed CCL2 expression. Monocytes (CD11b^{+}Ly6C^{high}) isolated from lungs of *Fuc-TVII*$^{-/-}$ and C56BL/6 mice had comparable levels of CCL2 expression that was about 4-fold higher than in circulating monocytes (Figure 5D). This finding indicates that monocytes recruited to the lungs express higher levels of CCL2 due to local activation upon tissue infiltration. We detected minimal expression of CCL2 in granulocytes (CD11b^{+}Ly6G^{high}Ly6C^{medium}) from both genotypes indicating that neutrophils do not significantly contribute to CCL2 presence in the lungs. This data provided evidence that the reduced ability of monocytes to be recruited to the lungs due to the absence of selectin ligands resulted in lower CCL2 levels in lungs of *Fuc-TVII*$^{-/-}$ mice. Finally, we analyzed whether reduced monocyte recruitment alters tumor cell survival in lungs of *Fuc-TVII*$^{-/-}$ mice. We observed higher apoptosis of tumor cells in lungs of *Fuc-TVII*$^{-/-}$ mice compared to C57BL/6 mice (Figure 5E-F), indicating that the lack of monocyte recruitment and therefore inefficient tumor cell extravasation led to tumor cell elimination.

Discussion

Inflammatory leukocytes at primary tumor and metastatic sites provide number of cytokines; growth factors and matrix-degrading enzymes thereby shaping the protumorigenic microenvironment [19; 28]. High infiltration of myeloid cells and inflammatory monocytes is often associated with enhanced tumor cell extravasation and malignant outgrowth [20; 22; 29]. Recruitment of myeloid cells to the metastatic sites has been shown to be dependent on the chemokine gradient (e.g. CCL2, CCL5) and on the capacity of leukocytes to adhere to activated endothelium in an L-selectin-dependent manner [10; 22; 29]. Previously, we observed enhanced expression of L-selectin ligands that was temporally and spatially restricted to the vicinity of metastatic tumor cells in the lungs [10]. Here we provided evidence that endogenous selectin ligands on monocytes are directly responsible for their specific recruitment to metastatic sites and contribute to tumor cell extravasation. This finding indicates that leukocytes recruitment to metastatic microenvironment is similar to inflammation, where the recruitment of leukocytes is also determined by selectin ligands on leukocytes [30]. Whether leukocytes bind to vascular selectins (P- and /or E-selectin) or through L-selectin of already adherent leukocytes requires further investigations.

We also tested whether Fuc-T7 deficiency has an effect on the primary tumor growth. MC-38GFP cells were injected subcutaneously into the flank of *Fuc-TVII*$^{-/-}$ and C57BL/6 mice. Interestingly, the primary tumor grew faster in *Fuc-TVII*$^{-/-}$ mice compared to C57BL/6 mice (Figure S7). This phenomenon is in line with previous findings showing an accelerated growth of primary tumors in selectin deficient mice [31]. Thus, endogenous selectin ligands restrict primary tumor growth but potentiate metastatic outgrowth at metastatic sites indicating that selectins may have spatially and temporally defined diverse functions. While we confirmed that selectin-

dependent recruitment of monocytes facilitates metastasis, the identity and function of leukocytes recruited at primary sites remains to be identified.

Neutrophils during cancer progression exert dual activities either promoting tumor cell growth or contributing to elimination of tumor cells depending on the polarization status and cellular context [32; 33]. Neutrophil-melanoma cell interactions promoted tumor cell retention within the lungs and contributed to transendothelial migration *in vitro* [32]. While in control mesothelioma tumors neutrophils contributed to cancer progression, the blockade of TGF-β resulted in recruitment of cytotoxic neutrophils leading to elimination of tumor cells [33]. Neutrophilia observed in *Fuc-TVII*$^{-/-}$ mice raised also the question whether neutrophils contribute to attenuated metastasis. Since increased association of neutrophils was observed 24 h p.i. in *Fuc-TVII*$^{-/-}$ mice, we depleted Ly6G+ cells using 1A8 antibody 6 h after tumor cell injection (Figure S8). After four weeks we observed partial rescue of metastasis in Ly6G-depleted animals, suggesting that increased neutrophil-tumor cell association contributes to elimination of tumor cells in *Fuc-TVII*$^{-/-}$ mice. These observations were further corroborated by detection of apoptotic tumor cells in lungs of *Fuc-TVII*$^{-/-}$ mice that was increased compared to C57BL/6 mice. Several studies reported that reduced recruitment of monocytes to tumor cells resulted in increased neutrophil association [22; 34]. Recruitment of monocytes to cancer cells was found to be primarily CCL2-dependent [22; 29; 34]. Thus, the ability of monocytes to inhibit neutrophil infiltration has been suggested [34]. This plasticity in leukocyte recruitment corresponds with our findings where reduced monocyte recruitment was accompanied with decreased CCL2 levels and resulted in enhanced neutrophil recruitment in *Fuc-TVII*$^{-/-}$ mice. However, intravenous injection of selectin-ligand-positive monocytes rescued metastasis. This finding confirmed the involvement of selectin-mediated recruitment of monocytes in metastasis. Thus, transient inhibition of selectin-selectin ligand-mediated interactions

reveals a potential intervention strategy to block metastatic dissemination at an early stage prior to tumor cell extravasation.

Acknowledgements

We thank Ruth Hillemann and Daniel Kull from Technical University Munich for preparing slides and immunohistochemical stainings. This work was supported by grants from Swiss National Foundation #31003A-133025 (to L.B.).

References

[1] R. Kannagi, Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. Glycoconj J 14 (1997) 577-584.

[2] Y.J. Kim, A. Varki, Perspectives on the significance of altered glycosylation of glycoproteins in cancer. Glycoconj J 14 (1997) 569-576.

[3] M. Tatsumi, A. Watanabe, H. Sawada, Y. Yamada, Y. Shino, H. Nakano, Immunohistochemical expression of the sialyl Lewis x antigen on gastric cancer cells correlates with the presence of liver metastasis. Clin Exp Metastasis 16 (1998) 743-750.

[4] S. Nakamori, M. Kameyama, S. Imaoka, H. Furukawa, O. Ishikawa, Y. Sasaki, T. Kabuto, T. Iwanaga, Y. Matsushita, T. Irimura, Increased expression of sialyl Lewisx antigen correlates with poor survival in patients with colorectal carcinoma: clinicopathological and immunohistochemical study. Cancer Res 53 (1993) 3632-3637.

[5] P. Grabowski, B. Mann, U. Mansmann, N. Lovin, H.D. Foss, G. Berger, H. Scherubl, E.O. Riecken, H.J. Buhr, C. Hanski, Expression of SIALYL-Le(x) antigen defined by MAb AM-3 is an independent prognostic marker in colorectal carcinoma patients. Int J Cancer 88 (2000) 281-286.

[6] I.P. Witz, The selectin-selectin ligand axis in tumor progression. Cancer Metastasis Rev 27 (2008) 19-30.

[7] H. Läubli, L. Borsig, Selectins promote tumor metastasis. Semin Cancer Biol 20 (2010) 169-177.

[8] R.P. McEver, Selectin-carbohydrate interactions during inflammation and metastasis. Glycoconj J 14 (1997) 585-591.

[9] Y.J. Kim, L. Borsig, N.M. Varki, A. Varki, P-selectin deficiency attenuates tumor growth and metastasis. Proc Natl Acad Sci U S A 95 (1998) 9325-9330.

[10] H. Läubli, J.L. Stevenson, A. Varki, N.M. Varki, L. Borsig, L-selectin facilitation of metastasis involves temporal induction of fut7-dependent ligands at sites of tumor cell arrest. Cancer Res 66 (2006) 1536-1542.

[11] A.M. Khatib, L. Fallavollita, E.V. Wancewicz, B.P. Monia, P. Brodt, Inhibition of hepatic endothelial E-selectin expression by C-raf antisense oligonucleotides blocks colorectal carcinoma liver metastasis. Cancer Res 62 (2002) 5393-5398.

[12] S. Hiratsuka, S. Goel, W.S. Kamoun, Y. Maru, D. Fukumura, D.G. Duda, R.K. Jain, Endothelial focal adhesion kinase mediates cancer cell homing to discrete regions of the lungs via E-selectin up-regulation. Proc Natl Acad Sci U S A 108 (2011) 3725-3730.

[13] G. Mannori, P. Crottet, O. Cecconi, K. Hanasaki, A. Aruffo, R.M. Nelson, A. Varki, M.P. Bevilacqua, Differential colon cancer cell adhesion to E-, P-, and L-selectin: role of mucin-type glycoproteins. Cancer Res 55 (1995) 4425-4431.

[14] Y.J. Kim, L. Borsig, H.L. Han, N.M. Varki, A. Varki, Distinct selectin ligands on colon carcinoma mucins can mediate pathological interactions among platelets, leukocytes, and endothelium. Am J Pathol 155 (1999) 461-472.

[15] L. Borsig, R. Wong, J. Feramisco, D.R. Nadeau, N.M. Varki, A. Varki, Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci U S A 98 (2001) 3352-3357.

[16] B. Shao, M.G. Wahrenbrock, L. Yao, T. David, S.R. Coughlin, L. Xia, A. Varki, R.P. McEver, Carcinoma mucins trigger reciprocal activation of platelets and neutrophils in a murine model of Trousseau syndrome. Blood 118 (2011) 4015-4023.

[17] G.S. Kansas, Selectins and their ligands: current concepts and controversies. Blood 88 (1996) 3259-3287.

[18] K. Ley, C. Laudanna, M.I. Cybulsky, S. Nourshargh, Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 7 (2007) 678-689.

[19] A. Mantovani, A. Sica, Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 22 (2010) 231-237.

[20] L. Zhao, S.Y. Lim, A.N. Gordon-Weeks, T.T. Tapmeier, J.H. Im, Y. Cao, J. Beech, D. Allen, S. Smart, R.J. Muschel, Recruitment of a myeloid cell subset (CD11b/Gr1(mid))via CCL2/CCR2 promotes thedevelopment of colorectal cancer liver metastasis. Hepatology 57 (2013) 829-839.

[21] B.Z. Qian, J. Li, H. Zhang, T. Kitamura, J. Zhang, L.R. Campion, E.A. Kaiser, L.A. Snyder, J.W. Pollard, CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 475 (2011) 222-225.

[22] M.J. Wolf, A. Hoos, J. Bauer, S. Boettcher, M. Knust, A. Weber, N. Simonavicius, C. Schneider, M. Lang, M. Sturzl, R.S. Croner, A. Konrad, M.G. Manz, H. Moch, A. Aguzzi, G. van Loo, M. Pasparakis, M. Prinz, L. Borsig, M. Heikenwalder, Endothelial CCR2 signaling induced by colon carcinoma cells enables extravasation via the JAK2-Stat5 and p38MAPK pathway. Cancer cell 22 (2012) 91-105.

[23] L. Borsig, R. Wong, R.O. Hynes, N.M. Varki, A. Varki, Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc Natl Acad Sci U S A 99 (2002) 2193-2198.

[24] H. Läubli, L. Borsig, Selectins as mediators of lung metastasis. Cancer Microenviron 3 (2010) 97-105.

[25] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25 (2001) 402-408.

[26] P. Maly, A. Thall, B. Petryniak, C.E. Rogers, P.L. Smith, R.M. Marks, R.J. Kelly, K.M. Gersten, G. Cheng, T.L. Saunders, S.A. Camper, R.T. Camphausen, F.X. Sullivan, Y. Isogai, O. Hindsgaul, U.H. von Andrian, J.B. Lowe, The alpha(1,3)fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. Cell 86 (1996) 643-653.

[27] J.W. Homeister, A.D. Thall, B. Petryniak, P. Maly, C.E. Rogers, P.L. Smith, R.J. Kelly, K.M. Gersten, S.W. Askari, G. Cheng, G. Smithson, R.M. Marks, A.K. Misra, O. Hindsgaul, U.H. von Andrian, J.B. Lowe, The alpha(1,3)fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. Immunity 15 (2001) 115-126.

[28] J.A. Joyce, J.W. Pollard, Microenvironmental regulation of metastasis. Nat Rev Cancer 9 (2009) 239-252.

[29] B.Z. Qian, J.W. Pollard, Macrophage diversity enhances tumor progression and metastasis. Cell 141 (2010) 39-51.

[30] M. Sperandio, C.A. Gleissner, K. Ley, Glycosylation in immune cell trafficking. Immunol Rev 230 (2009) 97-113.

[31] D. Taverna, H. Moher, D. Crowley, L. Borsig, A. Varki, R.O. Hynes, Increased primary tumor growth in mice null for beta3- or beta3/beta5-integrins or selectins. Proc Natl Acad Sci U S A 101 (2004) 763-768.

[32] S.J. Huh, S. Liang, A. Sharma, C. Dong, G.P. Robertson, Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. Cancer Res 70 (2010) 6071-6082.

[33] Z.G. Fridlender, J. Sun, S. Kim, V. Kapoor, G. Cheng, L. Ling, G.S. Worthen, S.M. Albelda, Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell 16 (2009) 183-194.

[34] J.C. Pahler, S. Tazzyman, N. Erez, Y.Y. Chen, C. Murdoch, H. Nozawa, C.E. Lewis, D. Hanahan, Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. Neoplasia 10 (2008) 329-340.

Figure Legends

Figure 1. Endogenous selectin ligands facilitate metastasis. A) Representative pictures of metastatic lungs from C57BL/6 (n=12) and *Fuc-TVII*$^{-/-}$ (n=7) mice 28 days p.i. with MC-38GFP cells. **B)** Quantification of metastatic foci 28 days p.i. **C)** Histological analysis of lungs from C57BL/6 and *Fuc-TVII*$^{-/-}$ mice 28 days p.i. with MC-38GFP cells. Scale bars are indicated. H&E: Hematoxylin & Eosin, Ki67: proliferation marker, F4/80: monocytes and macrophages, Ly6G: neutrophils, B220: B-cells, CD3: T cells. The insert in Ly6G^{+} cells shows magnification at the tissue-tumor interface at higher magnification. Scale bar = 100 μm. **D)** Macroscopy of lungs from C57BL/6 and *Fuc-TVII*$^{-/-}$ mice 14 days p.i. with Lewis lung carcinoma (3LL) cells. **E)** Quantification of tumor nodules (n = 4-5). **F)** Gating strategy for the quantification of GFP-expressing tumor cells in lung homogenates of C57BL/6 and *Fuc-TVII*$^{-/-}$ mice at various time-points. CD31^{+} endothelial cells (ECs) were used as an internal reference. **G)** Flow cytometry analysis of MC-38GFP cells in lung homogenates at different time points after injection. The amount of tumor cells is normalized to 20'000 ECs. *, $p < 0.05$.

Figure 2. Selectin ligands on hematopoietic cells are required for metastasis. A) Quantification of selectin ligands on monocytes (Ly6C^{hi} cells) from peripheral blood of C57BL/6, *FucTVII*$^{-/-}$ and chimeric mice: C57BL/6→*FucTVII*$^{-/-}$ or *FucTVII*$^{-/-}$ →C57BL/6 using L-selectin (n = 4). **B)** Quantification of Ly6G^{+} cells in the peripheral blood of control and chimeric mice (n = 4-5). **C)** Macroscopy of lungs from chimeric mice and **D)** quantification of tumor nodules in lungs of mice 28 days p.i. with MC-38GFP cells, respectively (n = 5/6). **, $p < 0.01$; ***, $p < 0.001$.

Figure 3. Fuc-TVII deficiency alters leukocyte recruitment to the lungs. A) Flow cytometry analysis of leukocytes from lung homogenates of untreated mice (C57BL/6, *Fuc-TVII*$^{-/-}$) and mice 14 h and 24 h p.i. with MC-38GFP cells (n = 4-6). The number of leukocytes (CD45^{+} cells) was normalized to 1'000 endothelial cells (ECs). Ly6G^{+}, Ly6C^{+} and F4/80^{+} cells are presented as percentage of CD45^{+} cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **B)** Representative confocal microscopy images of Ly6G^{+} cells (red) in lung sections of untreated C57BL/6 and *Fuc-TVII*$^{-/-}$ mice. Nuclei are stained in blue (DAPI). Scale bar: 50 μm.

Figure 4. The absence of endogenous selectin ligands reduces myeloid-tumor cell interactions. A) Quantification of the myeloid cell-tumor cell interactions in lungs of C57BL/6 and *Fuc-TVII*$^{-/-}$ mice at 14 h and 24 h p.i. with MC-38GFP cells by confocal microscopy. (n = 3). ***, $p < 0.001$. **B)** Representative confocal microscopy images of the contact of tumor cells (green) with F4/80^{+} or Ly6G^{+} cells (both in red) in C57BL/6 and *Fuc-TVII*$^{-/-}$ lungs at 24 h p.i. Nuclei (blue) are stained with DAPI. Scale bar: 10 μm.

Figure 5. Selectin ligand-expressing monocytes rescued metastasis in *Fuc-TVII*$^{-/-}$ mice. Adoptive transfer of monocytes (monos) isolated from bone marrow of C57BL/6 and *Fuc-TVII*$^{-/-}$ mice that were intravenously injected into *Fuc-TVII*$^{-/-}$ mice 6 h p.i. of MC-38GFP cells. **A)** Macroscopy of lungs from *Fuc-TVII*$^{-/-}$ mice that were injected with C57BL/6 or *Fuc-TVII*$^{-/-}$ monocytes 28 days p.i. **B)** Quantification of tumor nodules in lungs at day 28 (n = 7-10). **C-D)** CCL2 expression in the whole lungs (**C**) and monocytes (Ly6C^{high}) isolated from lungs (**D**) of mice i.v. injected with MC-38GFP cells after 14 h and 24 h, respectively. Data represents two independent experiments. Expression levels were analyzed by real time-PCR and normalized to

GAPDH. Purified blood monocytes (Ly6C^{high}) from untreated mice were used as controls. Data are presented as $2^{-\Delta\Delta ct}$. **E)** Caspase 3 stained lungs section of C57Bl/6 and *Fuc-TVII*$^{-/-}$ mice 24 h after MC-38GFP injection. Three mouse specimens were evaluated. Representative images of Caspase3^{+} tumor cell in *Fuc-TVII*$^{-/-}$ mice and Caspase3^{-} tumor cells in C57BL/6 lungs are shown. **F)** Analysis of GFP^{+} tumor cells in lungs of mice in parallel to Caspase 3 staining. *, $p < 0.05$, ***, $p < 0.001$.

Figure 1

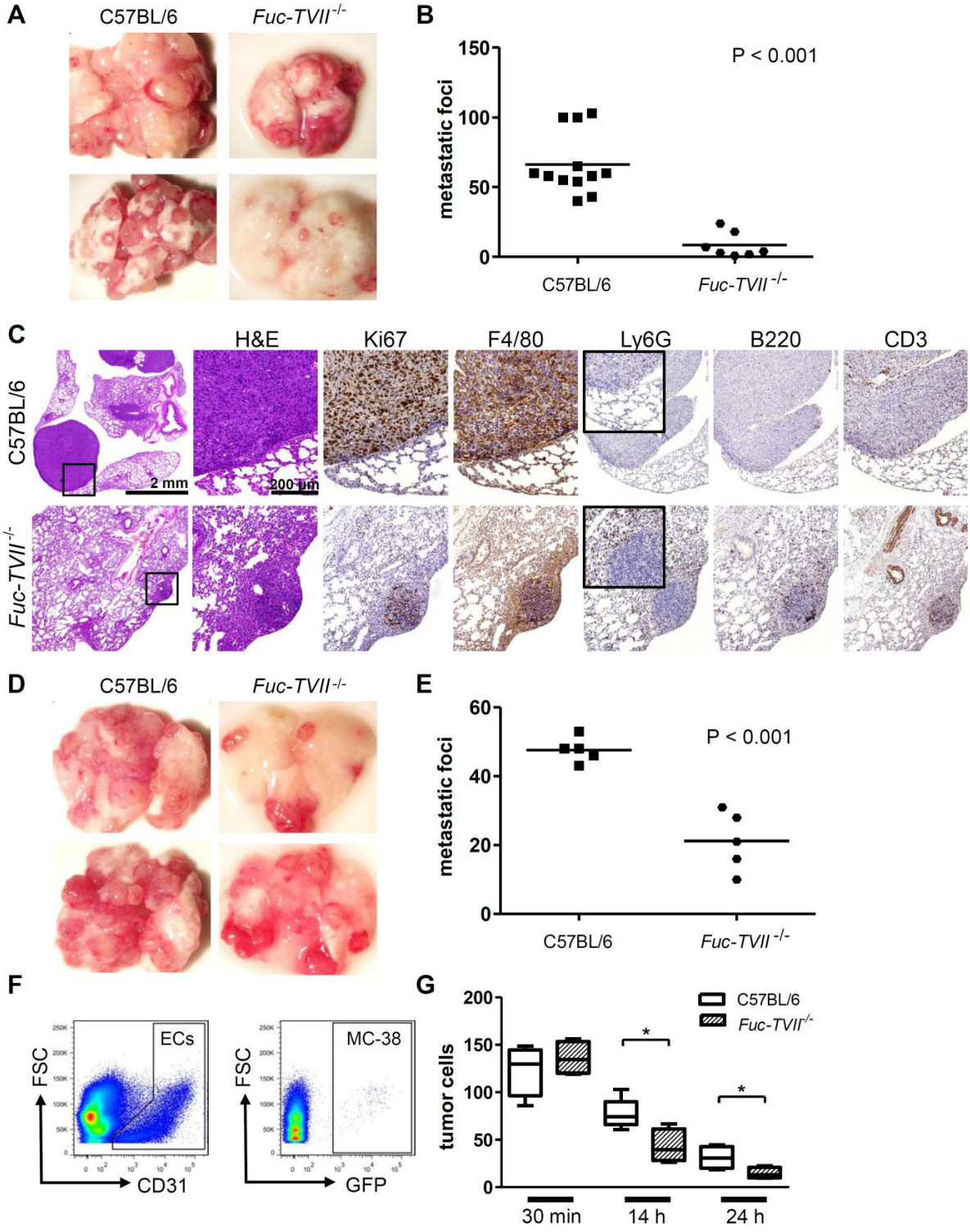

A
C57BL/6
Fuc-TVII -/-
B
P < 0.001
metastatic foci
150
100
50
0
C57BL/6
Fuc-TVII -/-
C
H&E
Ki67
F4/80
Ly6G
B220
CD3
C57BL/6
Fuc-TVII -/-
2 mm
200 µm
D
C57BL/6
Fuc-TVII -/-
E
P < 0.001
metastatic foci
60
40
20
0
C57BL/6
Fuc-TVII -/-
F
ECs
FSC
CD31
MC-38
FSC
GFP
G
C57BL/6
Fuc-TVII -/-
tumor cells
200
150
100
50
0
*
*
30 min
14 h
24 h

Figure 2

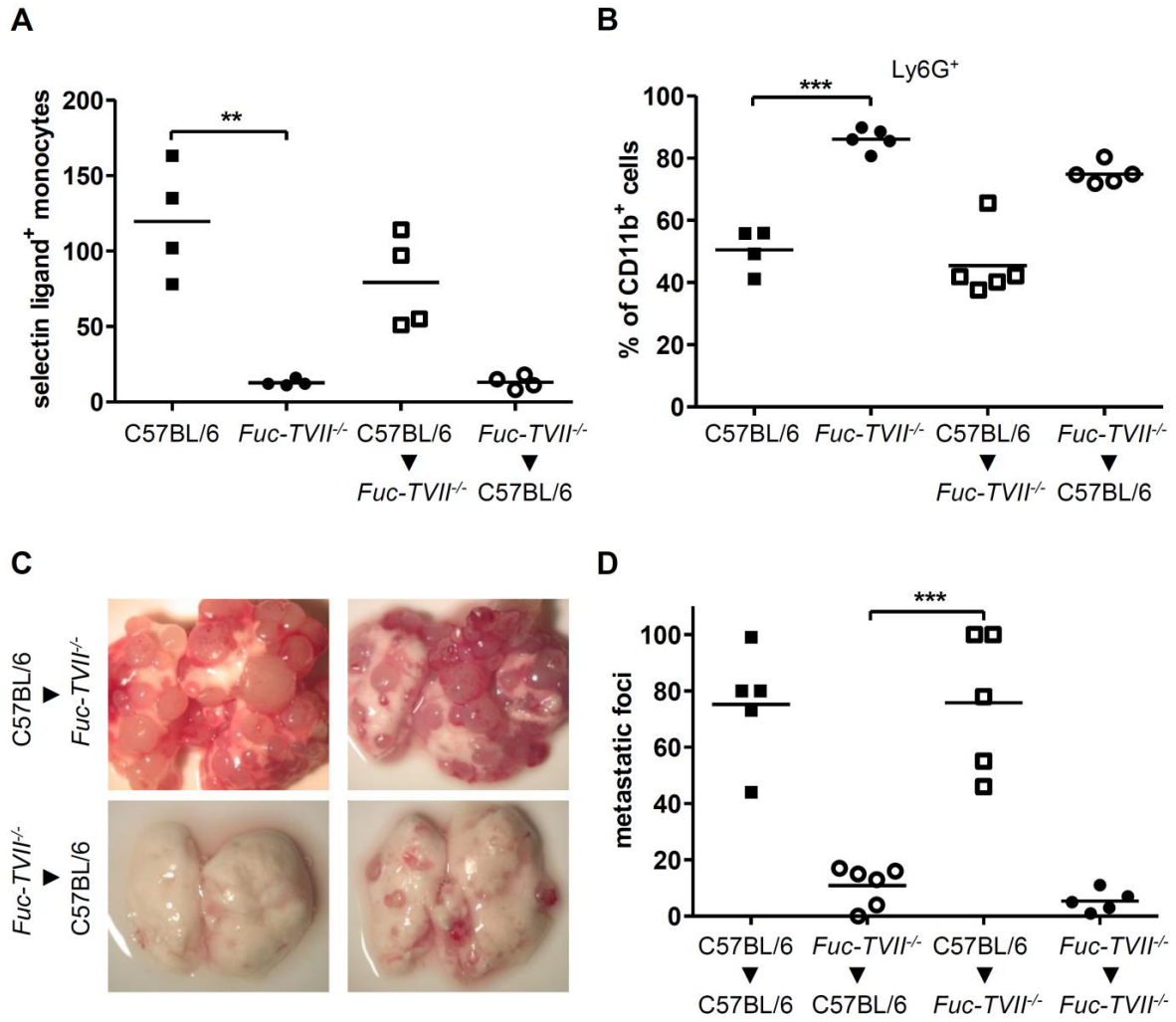

A
selectin ligand+ monocytes
200
150
100
50
0
**
C57BL/6
Fuc-TVII-/-
C57BL/6
▼
Fuc-TVII-/-
Fuc-TVII-/-
▼
C57BL/6
B
Ly6G+
***
% of CD11b+ cells
100
80
60
40
20
0
C57BL/6
Fuc-TVII-/-
C57BL/6
▼
Fuc-TVII-/-
Fuc-TVII-/-
▼
C57BL/6
C
C57BL/6
▼
Fuc-TVII-/-
Fuc-TVII-/-
▼
C57BL/6
D
***
metastatic foci
100
80
60
40
20
0
C57BL/6
▼
C57BL/6
Fuc-TVII-/-
▼
C57BL/6
C57BL/6
▼
Fuc-TVII-/-
Fuc-TVII-/-
▼
Fuc-TVII-/-

Figure 3

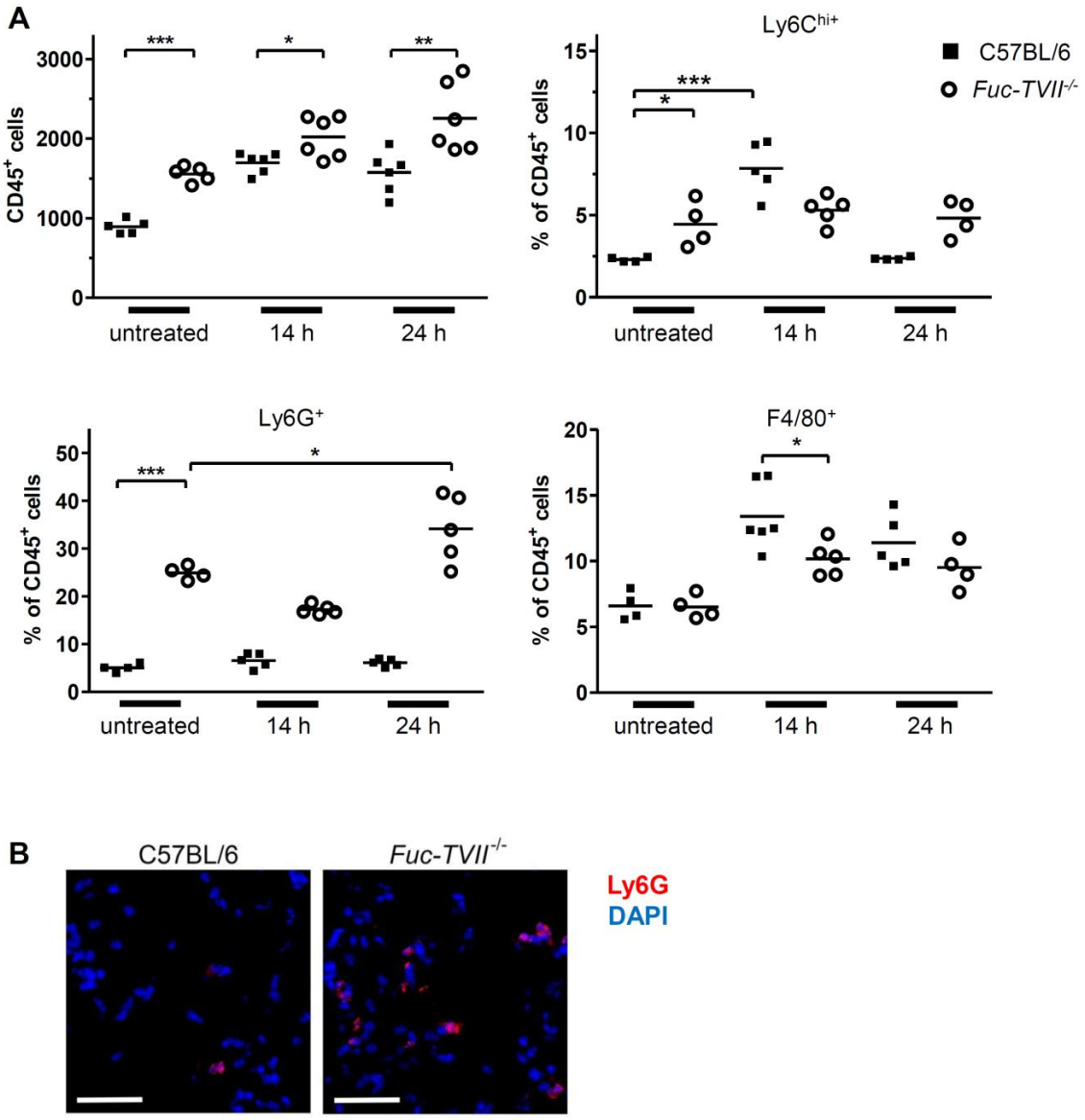

A
CD45+ cells
3000
2000
1000
0
***
*
**
untreated
14 h
24 h
Ly6Chi+
% of CD45+ cells
15
10
5
0
***
*
C57BL/6
Fuc-TVII-/-
Ly6G+
50
40
30
20
10
F4/80+
20
B
C57BL/6
Fuc-TVII-/-
Ly6G
DAPI

Figure 4

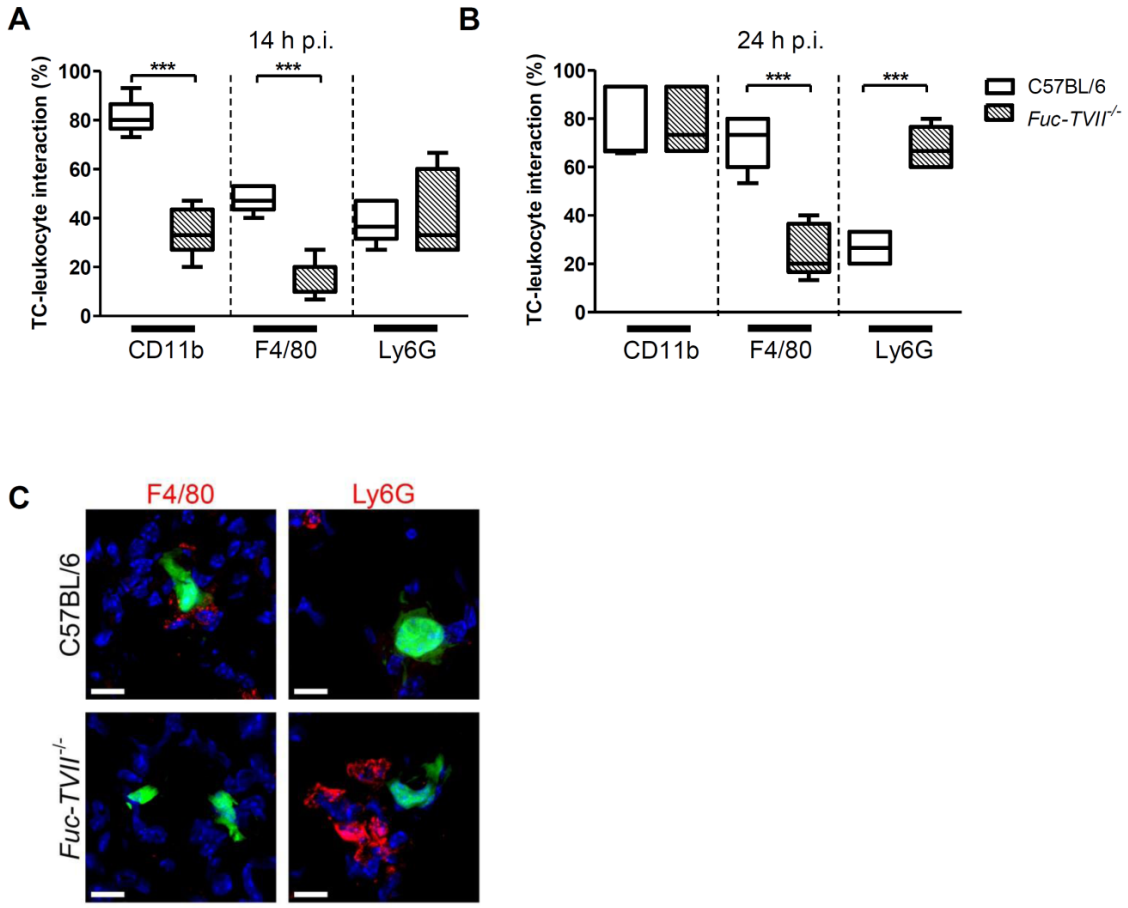
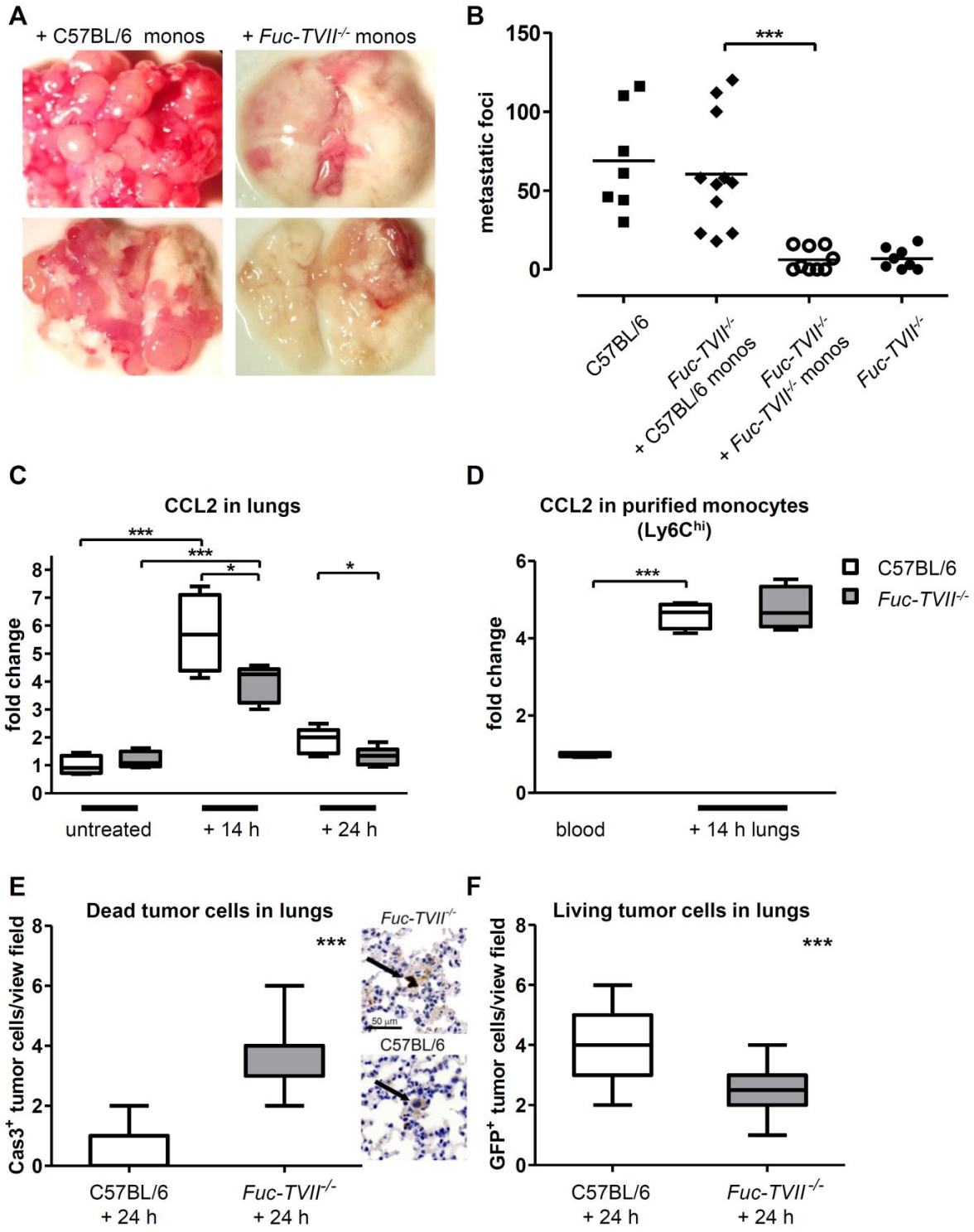

A
14 h p.i.
TC-leukocyte interaction (%)
100
80
60
40
20
0
***
***
CD11b
F4/80
Ly6G
B
24 h p.i.
TC-leukocyte interaction (%)
100
80
60
40
20
0
***
***
CD11b
F4/80
Ly6G
C57BL/6
Fuc-TVII-/-
C
F4/80
Ly6G
C57BL/6
Fuc-TVII-/-

Figure 5


A
+ C57BL/6 monos
+ Fuc-TVII-/- monos
B
metastatic foci
150
100
50
0
***
C57BL/6
Fuc-TVII-/- + C57BL/6 monos
Fuc-TVII-/- + Fuc-TVII-/- monos
Fuc-TVII-/-
C
CCL2 in lungs
fold change
untreated
+ 14 h
+ 24 h
D
CCL2 in purified monocytes (Ly6Chi)
C57BL/6
Fuc-TVII-/-
fold change
blood
+ 14 h lungs
E
Dead tumor cells in lungs
Cas3+ tumor cells/view field
Fuc-TVII-/-
50 µm
C57BL/6
C57BL/6 + 24 h
Fuc-TVII-/- + 24 h
F
Living tumor cells in lungs
GFP+ tumor cells/view field
C57BL/6 + 24 h
Fuc-TVII-/- + 24 h

Supplementary material:

Selectin ligands on leukocytes are required for monocyte recruitment and adhesion at metastatic sites

Alexandra Hoos, Darya Protsyuk, Lubor Borsig

Inventory of Supplemental Information

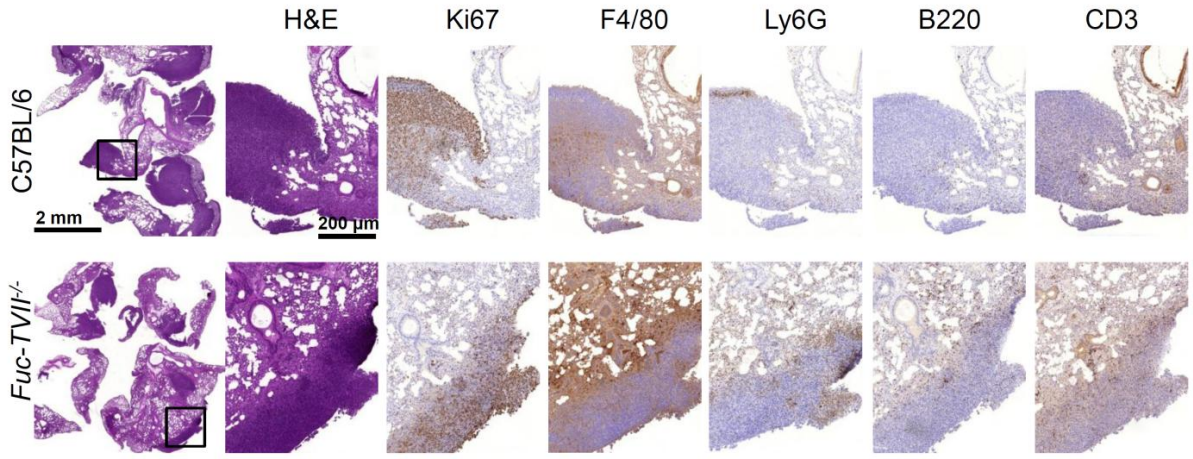


Figure S1. Endogenous selectin ligands attenuate metastasis of 3LL cells.
Histological analysis of tumor bearing lungs from C57BL/6 and *Fuc-TVII*$^{-/-}$ mice 14 days p.i. Scale bars as indicated. H&E: Hematoxylin & Eosin, Ki67: proliferation marker, F4/80: monocytes and macrophages, Ly6G: neutrophils, B220: B-cells, CD3: T cells. Increased infiltration of CD3^{+} and B220^{+} cells observed in MC-38GFP tumors was not observed in 3LL tumors indicating that tumor cells might induce specific inflammatory responses.

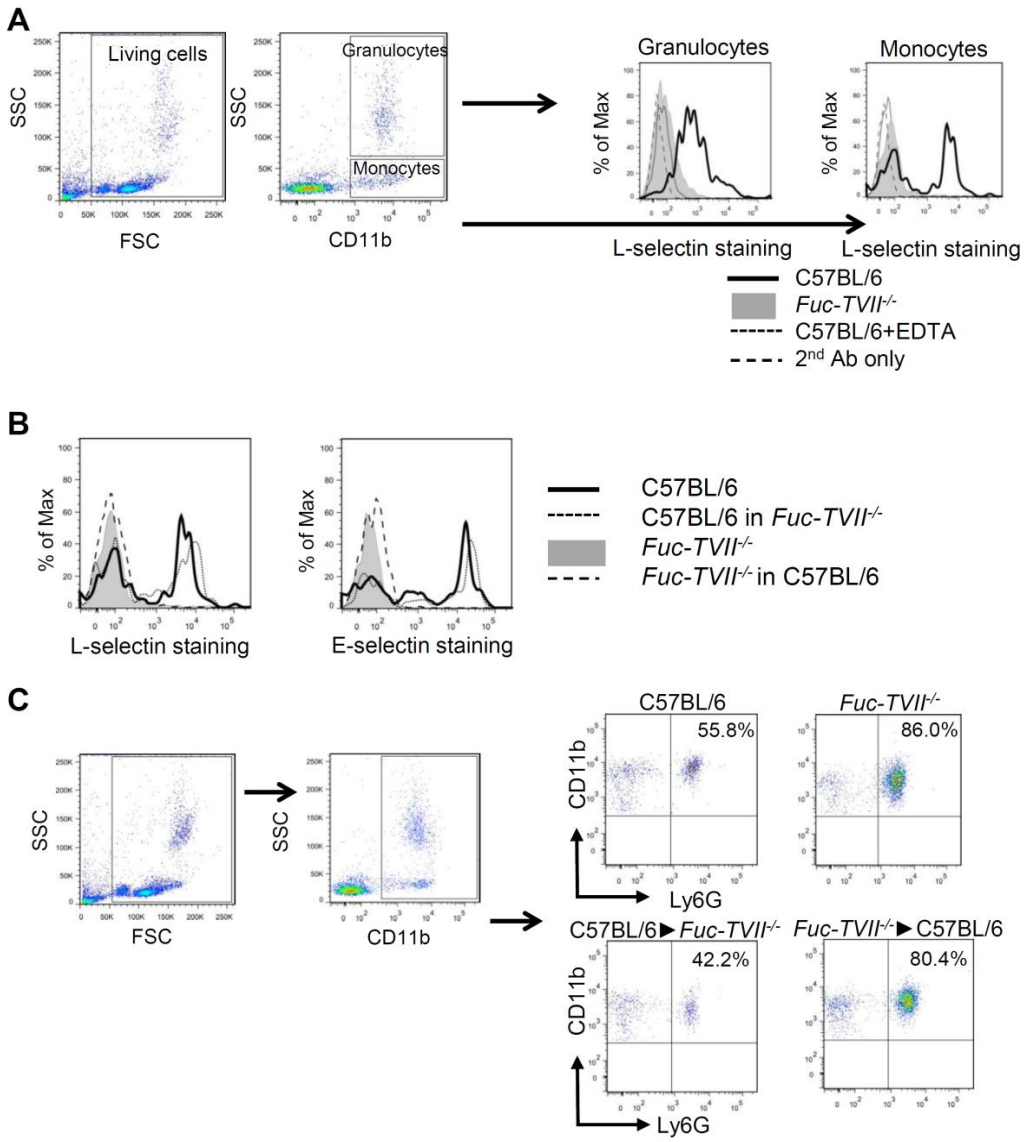


Figure S2. Reconstitution efficacy of chimeric mice. A) Gating strategy for the analysis of selectin ligand expression on myeloid cells in the peripheral blood. **B)** Representative histograms of L- and E-selectin ligand expression on monocytes from the blood of control and chimeric mice. **C)** Gating strategy for leukocytes. Blood from control and chimeric mice was analyzed by flow cytometry. The number of $Ly6G^+$ cells was quantified.

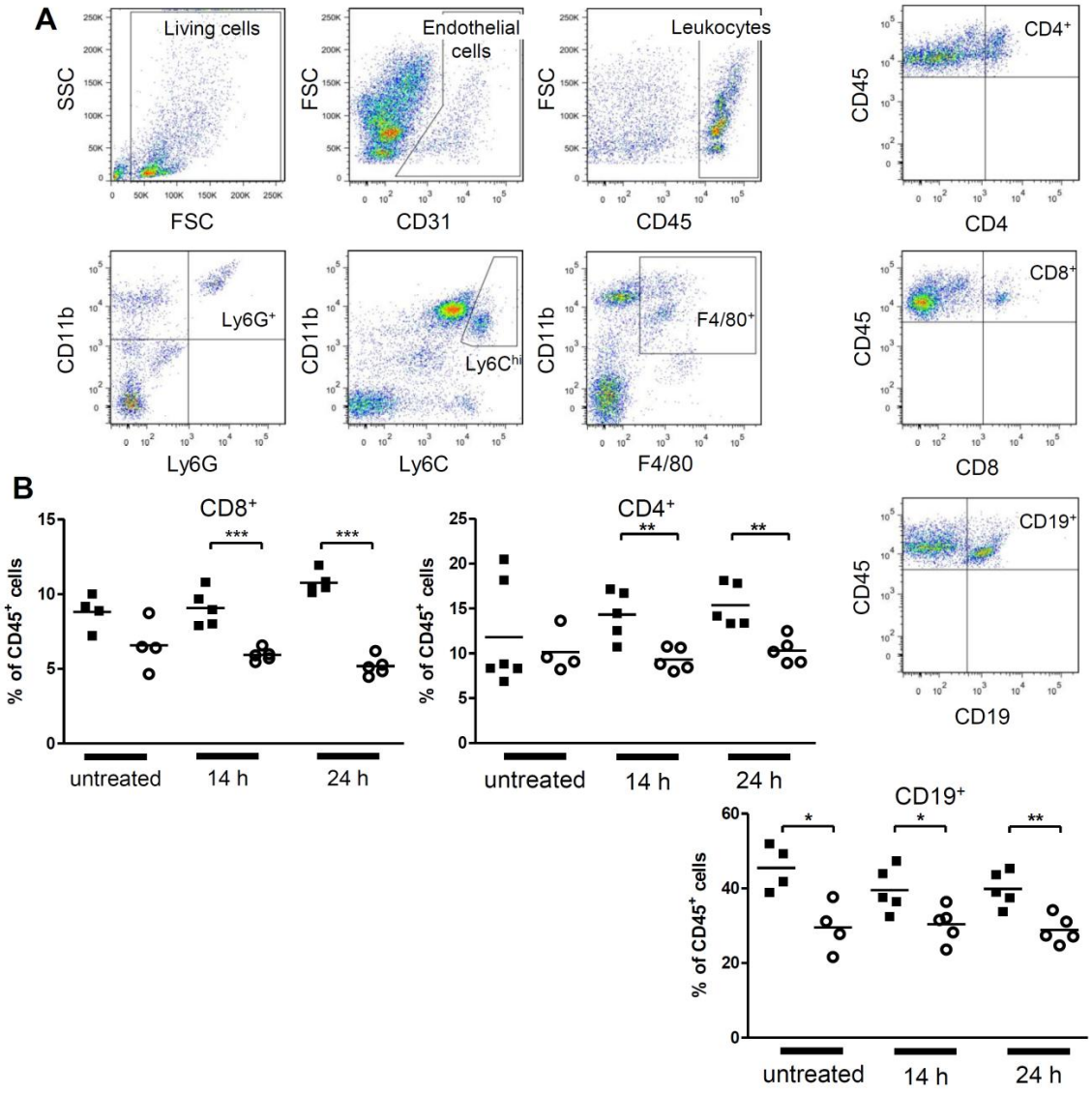


Figure S3. Analysis of infiltrating leukocytes to the lungs. A) Gating strategy. Leukocytes were gated based on CD45-APC-Cy7 staining and endothelial cells (CD31-FITC) were used as an internal control. Leukocyte subpopulations were characterized with the following mAbs for Ly6G, Ly6C, F4/80, CD4, CD8, CD19. **B)** Reduced lymphocyte infiltration to the lungs of *FucT-VII* deficient mice. Leukocytes (CD45+ cells) were normalized to 1'000 endothelial cells for quantification. Flow cytometry analysis of $CD4^+$, $CD8^+$ and CD 19^+ cells in untreated lungs of C57BL/6 and *Fuc-TVII*$^{-/-}$ mice and mice treated with MC-38GFP cells 14 h and 24 h p.i. (n = 4-6). *, $p < 0.05$; **, $p < 0.01$; ***. $p < 0.001$.

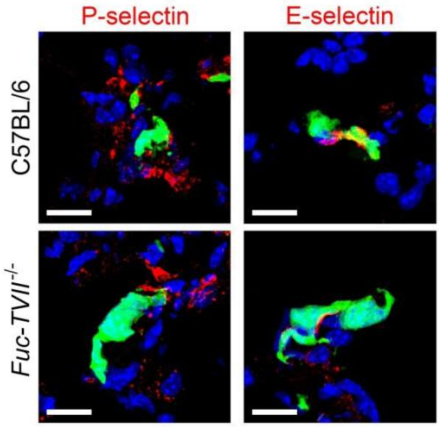


Figure S4. Expression of E- and P-selectin in the vicinity of MC-38GFP cells is not affected in *Fuc-TVII*$^{-/-}$ mice. Representative confocal microscopy images of E- and P-selectin expression (red) in lung sections from C57BL/6 and *Fuc-TVII*$^{-/-}$ mice injected with MC-38GFP cells (green) at 6 h p.i. Nuclei are stained with DAPI (blue). Scale bar:15 μm.

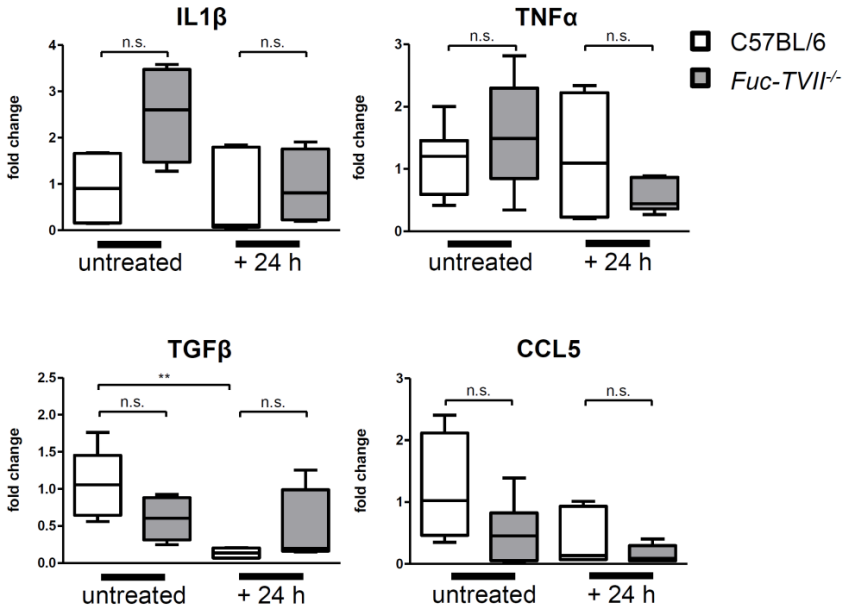


Figure S5. The absence of endogenous selectin ligands has no effect on expression levels of inflammatory cytokines. Expression levels of selected cytokines in lungs of mice in response to MC-38GFP injection 24 h p.i. (n = 3). The expression levels were analyzed by real time-PCR and normalized to GAPDH. Data are presented as $2^{-\Delta\Delta ct}$. **, $p < 0.01$, n.s. = not significant.

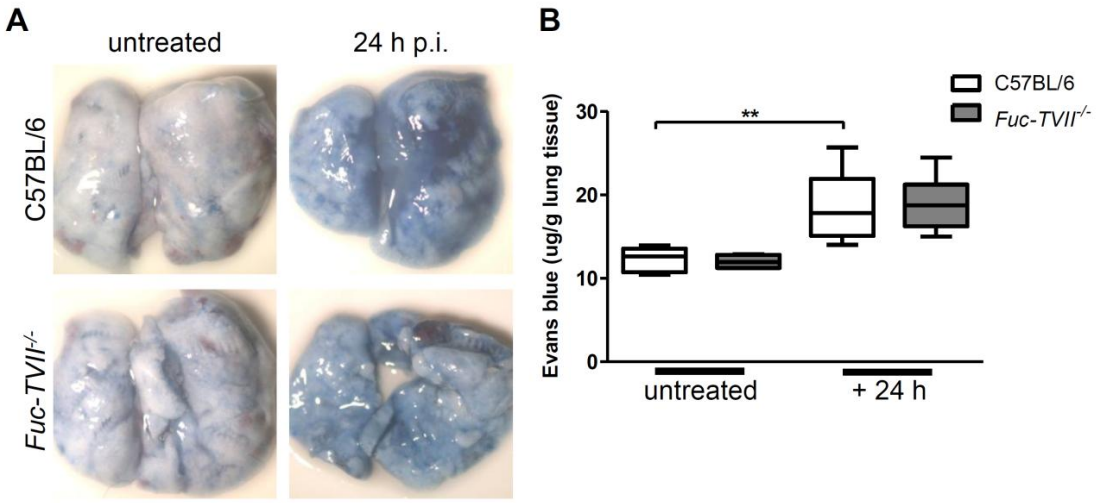


Figure S6. Analysis of lung vascular permeability. Mice were intravenously injected with MC-38GFP cells. After 24 h 2mg of Evans blue was injected i.v. and lungs were perfused 30 min later. **A)** Macroscopy of lungs in C57BL/6 and *Fuc-TVII*$^{-/-}$ mice with naïve or MC-38GFP injected lungs. **B)** Spectrophotometric quantification of Evans blue extracted from lung tissues. **, $p < 0.01$.

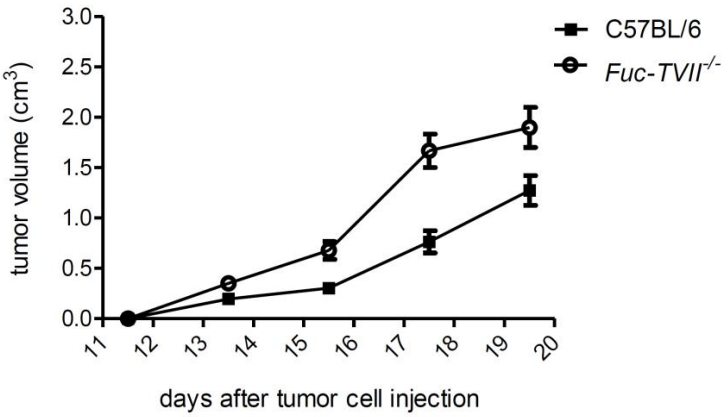


Figure S7. *Fuc-TVII*$^{-/-}$ mice accelerate primary tumor growth. MC-38GFP cells were injected into the flank of C57BL/6 and *Fuc-TVII*$^{-/-}$ mice (n = 3) . The tumor growth was monitored every 2-3 day. Tumor dimensions length (L) and width (W) were measured and the tumor volume was calculated according to the formula $V=\pi LW2/6$.

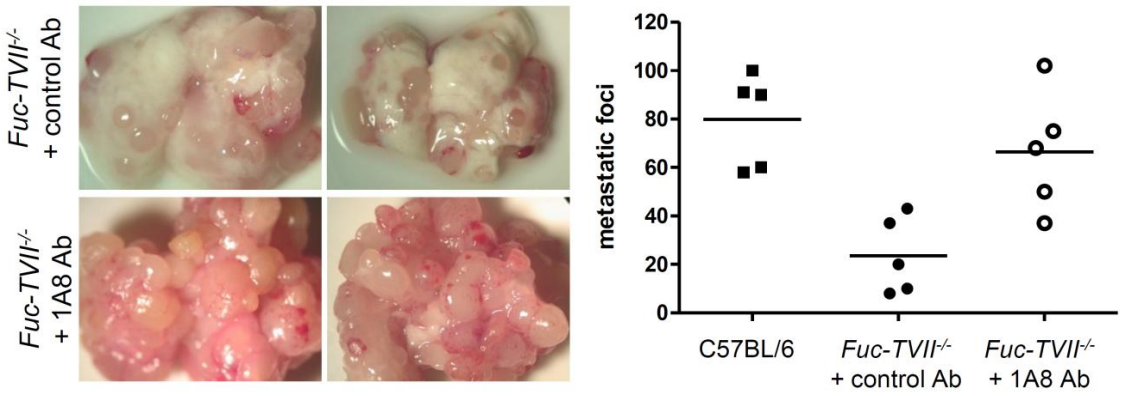


Figure S8. Transient depletion of neutrophils rescued metastasis in *Fuc-TVII*$^{-/-}$ mice. Mice were intravenously injected with 1A8 or isotype control and 6 h later with MC-38GFP cells. **A)** Macroscopy of lungs from *Fuc-TVII*$^{-/-}$ mice 28 days p.i. with MC-38GFP cells. **B)** Quantification of metastatic foci 28 days p.i.

Table S1.

Primers	Sequences 5`-3`	Fragment size (bp.)	Tm (°C)
CCL2	TTA ACG CCC CAC TCA CCT GC TGG GGT CAGCAC AGA CCT CTC	121	60
CCL5	TGC TGC TTT GCC TAC CTC ACA CTT GGC GGT TCC TTC	121	60
IL1β	TCC AGG ATG AGG ACA TGA GCA C GAA CGT CAC ACA CCA GCA GGT TA	105	60
TNFα	CCC TCA CAC TCA GAT CAT CTT CT GCT ACG ACG TGG GCT ACA G	61	60
TGFβ1	GCT GAC CCC CAC TGA TAC GC GTT TGG GGC TGA TCC CGT TGA	170	60
CXCL1	CCG AAG TCA TAG CCA CAC TCA A GCA GTC TGT CTT CTT TCT CCG TTA	128	59
CXCL2	GAG CTT GAG TGT GAC GCC CCC AGG GTT AGC CTT GCC TTT GTT CAG TAT C	148	60
CXCL5	GGT CCA CAG TGC CCT ACG GCG AGT GCA TTC CGC TTA	146	59

Supplemental Experimental Procedures

Detection of selectins: Frozen lung sections (8-10 µm) were prepared from mice i.v. injected with MC-38GFP cells as described previously [1], and were stained with mAb against murine P- and E-Selectin (BD) followed by goat anti-rat-Alexa568 Ab (Gibco). Images were acquired on a SP2 confocal microscope (Leica) of a total of 5 µm in a z-axis and analyzed with Imaris Software (Bitplane). Granulocytes were detected with anti-Ly6G Ab (BD) in the lungs of C57BL/6 and *Fuc-TVII*$^{-/-}$ mice and visualized with goat anti-rat-Alexa568 (Gibco).

Vascular Permeability assay: Permeability of the lung microvasculature was determined using the Evans blue dye as described previously [2]. Briefly, 24 h after tumor cell injection, 2 mg of Evans blue were i.v. injected and mice terminated 30 min later. Lungs were perfused with PBS, dissected, photographed and homogenized. Evans blue was extracted by incubation with formamide at 60°C for 18 h and the amount was measured with a spectrophotometer (absorbance at 620 nm).

Neutrophil depletion: Transient neutrophil depletion was achieved using a single intravenous injection of 50 µg rat-anti-Ly6G antibody (Cl. 1A8) in 100 µl HBSS. Control animals were injected with 50 µg isotype-control Ab (Cl. 2A3) both obtained from BioXCell, NH, U.S.A. MC-38GFP cells were intravenously injected 6 h after 1A8 or isotype control Ab injection. Mice were euthanized after 28 days and metastasis was quantified as described above.

References

[1] L. Borsig, R. Wong, J. Feramisco, D.R. Nadeau, N.M. Varki, A. Varki, Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci U S A 98 (2001) 3352-3357.

[2] M.J. Wolf, A. Hoos, J. Bauer, S. Boettcher, M. Knust, A. Weber, N. Simonavicius, C. Schneider, M. Lang, M. Sturzl, R.S. Croner, A. Konrad, M.G. Manz, H. Moch, A. Aguzzi, G. van Loo, M. Pasparakis, M. Prinz, L. Borsig, M. Heikenwalder, Endothelial CCR2 signaling induced by colon carcinoma cells enables extravasation via the JAK2-Stat5 and p38MAPK pathway. Cancer cell 22 (2012) 91-105.

DISCUSSION

The tumor microenvironment modulates the behaviour of cancer cells at both the primary and metastatic sites.[1] Secretion of cytokines together with the recruitment of monocytes has been linked to cancer progression and enhanced metastasis.[2-5]

1. Endothelial CCR2 - regulator of tumor cell extravasation

Chemokine receptors expressed on tumor cells were shown to affect the spread of tumor cells to particular organs.[2,4] For example, $CXCR4^+$ tumor cells metastasized preferentially to organs with high SDF-1 expression like bone, lungs and liver.[6] Similarly, CXCR3-mediated signaling in cancer cells facilitated lung metastasis in a murine model of metastatic breast cancer.[7] In another model the recruitment of myeloma cells to the bone marrow was CCR2 dependent.[8] The inhibition of chemokine receptors abolished metastasis to the target organs, indicating the chemokine-chemokine receptor axis as a possible explanation for the tissue tropism typical of some tumor cell lines.[6-8] In addition, enhanced CCR2 expression in prostate cancer was associated with tumor metastasis to bone.[9] CCR2 knockdown in prostate cancer cells diminished cell invasion and proliferation, emphasizing the role of chemokine receptors in tumor development.[9]

The role of host-derived chemokine receptors in metastasis is under investigation. The recruitment of MDSCs into the tumor was shown to depend on CCR2, suggesting a role for chemokine receptors in tumor immunosuppression.[10] Infiltration of $CCR2^+$ inflammatory monocytes into primary tumor correlates with tumor progression and increased metastatic spread.[11-13] Recently, CXCR2 expression in the endothelial compartment was reported to contribute to tumor development and metastasis.[14] CXCR2 was shown to mediate tumor growth and angiogenesis in a murine model of lung cancer. While spontaneous lung metastasis was markedly reduced in *CXCR2*$^{-/-}$ mice, the mechanism by which CXCR2 attenuated this process remains unclear.[14]

High levels of CCL2 in tumors correlates with tumor progression and metastasis.[5,15-17] Therefore, we examined whether CCR2, a receptor for CCL2, contributes to tumor cell extravasation and metastasis, respectively. Upon injection of MC-38GFP and 3LL cells,

metastasis was markedly reduced in *Ccr2*$^{-/-}$ mice. Primary tumor growth was not affected in *Ccr2*$^{-/-}$ mice, arguing against the possibility that the observed reduction in metastasis is the result of tumor growth rate.[18] Using chimeric, transgenic, and knockout mice, we could determine the contribution of the endothelial-derived CCR2 and of the CCR2 expressed on hematopoietic cells (discussed later) to metastasis. CCR2 expression restricted to endothelial cells (Tie2CCR2/*Ccr2*-/- mice) partially restored metastasis, highlighting the role of stromal CCR2 as a facilitator of metastasis. I could demonstrate *in vitro* and *in vivo*, that tumor cells were unable to migrate through a CCR2-deficient endothelial barrier. Thus, the presence of CCR2 on the endothelium is a crucial factor for tumor cell extravasation. Interestingly, the absence of CCR2 on endothelial cells did not affect the transmigration ability of monocytes, suggesting that transmigration per se, is not limited due to CCR2 deficiency. How can endothelial CCR2 regulate tumor cell extravasation? The endothelial chemokine receptor CXCR2 has been shown to induce vascular permeability in an LPS-induced model of inflammation.[19] Vascular destabilization in the premetastatic phase is a prerequisite for metastasis.[20] Indeed, vascular permeability in lungs of C57BL/6 mice was increased whereas the vascular integrity in CCR2 deficient lungs remained unchanged after tumor cell injection. Similarly, a *CCR2*$^{-/-}$ endothelial monolayer failed to retract in response to tumor cells. Taken together, these data provided evidence that tumor cells stimulate endothelial CCR2 to open the endothelial barrier, enabling extravasation of tumor cells.

Activation of endothelial cells implicates several signaling pathways in the process of leukocyte diapedesis during inflammation.[21,22] For instance, p38MAPK stimulated neutrophil emigration in response to keratinocyte-derived cytokine KC.[23] Cytokine signaling through JAK1-STAT3 regulated actomyosin contractility in tumor cells and stroma.[24] Increased activity of ERK and p38MAPK was associated with enhanced transendothelial migration of colon cancer cells.[25] Since, some of these pathways act downstream of CCR2[26,27], I tested a panel of inhibitors to determine which signaling pathways are involved in CCR2-dependent transmigration of tumor cells. Inhibition of JAK2, Stat5 and p38MAPK blocked the transmigration of tumor cells, whereas inhibitors for PI3K, Rac and Stat5 did not prevent this

process. The absence of phosphorylated JAK2, STAT5 and p38MAPK in lung homogenates from MC-38-injected *Ccr2*$^{-/-}$ mice compared to C57BL/6 mice, confirmed that CCR2 expression is crucial for activation of these signaling cascades. Furthermore, administration of JAK2 and p38MAPK inhibitors led to reduced vascular permeability in C57BL/6 lungs upon tumor cell injection. Previously observed increases in vascular permeability due to p38MAPK activation are in line with our findings.[25] Moreover, blocking of signaling pathways downstream of CCR2 significantly reduced metastasis, supporting the notion that endothelial CCR2 mediates colon cancer extravasation. This study demonstrates for the first time that a stromal chemokine receptor controls metastasis by promoting tumor cell extravasation.

2. Tumor cell-derived CCL2 - inducer of vascular permeability

High levels of CCL2 have been detected in tumors of colon, breast, liver, cervix and prostate cancer patients.[5,15-17] Our analysis of primary colon tumors (UICC stages I–IV) confirmed the link between CCL2 up-regulation in stage IV colon carcinoma and metastatic capacity. Several studies reported distinct CCL2 functions in tumor progression.[28] For example, CCL2 acts on cells within the tumor microenvironment such as endothelial cells and monocytes, which in turn, promote tumor development and metastasis.[12,29] It has been shown that CCL2 contributes to angiogenesis.[29,30] The CCL2-CCR2 axis has also been shown to facilitate metastasis through the recruitment of monocytes.[11-13] Inhibition of CCL2-CCR2 signaling abolished the recruitment of inflammatory monocytes, attenuated metastasis and prolonged the survival of tumor-bearing mice.[12] However the source of the CCL2 production within the tumor microenvironment remained undefined.

In order to determine the role of tumor and host-derived CCL2 in the process of CCR2-dependent tumor cell extravasation, I knocked down CCL2 expression in two different syngeneic tumor cells lines (MC-38GFP and 3LL). Silencing of CCL2 in tumor cells blocked tumor cell migration and impeded vascular permeability in C57BL/6 mice, indicating the essential role of tumor-derived CCL2 in these steps of metastasis. CCL2 deficient tumor cells could not form lung metastases, corroborating our findings that CCL2 secreting tumor cells

exploit endothelial CCR2 signaling for efficient tumor cell extravasation. Moreover, tumor cells with normal CCL2 expression induced similar vascular permeability in *Ccl2*$^{-/-}$ mice compared to C57BL/6 mice, highlighting that tumor cell-derived CCL2 is sufficient for the initiation of tumor cell extravasation. However, reduced metastasis in *Ccl2*$^{-/-}$ mice suggests that host-derived CCL2 also contributes to recruitment of inflammatory monocytes and thereby metastasis.[12] Next, performing co-injection experiments of MC-38GFP^{CCL2kd}/MC-38 cells we could demonstrate that close contact between CCL2^{+} tumor cells and CCR2^{+} endothelium is necessary for efficient tumor cell extravasation and metastasis. Thus, we identified a novel role for tumor cell-derived chemokines in metastasis which goes beyond the attraction of inflammatory cells.

Interestingly, melanoma cell line B16-BL6 expresses only a little CCL2 and could extravasate and metastasize independently of endothelial CCR2 expression. This leaves the question open whether different tumor cells exploit other endothelial chemokine receptors for extravasation.

3. Tumor cell-derived CCL2 - major attractor for inflammatory monocytes

In accordance with other studies, we could confirm the significance of CCR2^{+} expressing monocytes in metastasis. Silencing of CCR2 in monocytes led to reduced recruitment of monocytes to the primary tumor.[31] In our study, CCR2 depletion restricted to monocytes in LysMCre*Ccr2*$^{loxP/loxP}$ mice strongly affected monocyte recruitment and interaction with tumor cells and consequently reduced metastasis. Moreover, the number of metastases in *Ccr2*$^{-/-}$/C57BL/6 chimeric mice was considerably lower than in C57BL/6 mice, corroborating the role of CCR2^{+} monocytes as facilitators of metastasis. CCR2-expressing monocytes deliver molecules like VEGF, MMP9, IL10 and TGFβ that promote tumor cell survival and extravasation.[32,33] Consistent with this, I observed, that monocytes fostered the migration of tumor cells through the endothelial barrier. However, the migration of tumor cells through the CCR2 deficient endothelial monolayer was blocked, even during co-incubation with

monocytes. This highlights that both, $CCR2^+$ monocytes and $CCR2^+$ endothelial cells are needed for efficient tumor cell extravasation.

Tumor cells devoid of CCL2 failed to recruit and interact with inflammatory monocytes, suggesting that tumor cell-derived CCL2 is essential for the retention of $CCR2^+$ monocytes at metastatic sites. In agreement with other studies[18,34] increased association of neutrophils ($Ly6G^+$ cells) with tumor cells in the absence of monocytes was observed in *CCR2*$^{-/-}$ mice, implying that neutrophils might also attenuate metastasis. In addition, the effect of $CCR2^+$ MDSCs on metastasis in response to tumor-derived CCL2 cannot be excluded.[10]

4. Endogenous selectin ligands - facilitators of metastasis

Inflammatory leukocytes act as powerful tumor promoters by secreting various factors like matrix degrading enzymes, growth factors, cytokines and chemokines.[1,32] Localized at the target site, monocytes exert their pro-tumorigenic functions, enabling tumor cell survival, extravasation and growth.[32,33] Since leukocyte homing is regulated by concerted action of adhesion molecules and chemokines[21,22], we next investigated the role of endogenous selectin ligands in metastasis. Although selectin ligands expressed on tumor cells are commonly recognized to facilitate metastasis[35-37], the relevance of endogenous selectin ligands expressed on leukocytes and endothelial cells in this process is not known.

Evidence from our laboratory showed that L-selectin on leukocytes facilitated metastatic spread in a murine model of experimental lung metastasis.[38] The expression of endogenous L-selectin ligands was transiently increased in the vicinity of tumor cells.[38] This temporal appearance correlated with enhanced tumor load, suggesting that endogenous selectin ligands might augment metastasis. In this study we investigate the molecular mechanism by which endogenous selectin ligands promote metastasis.

4.1. Endogenous selectin ligands capture monocytes at metastatic sites

Absence of endogenous selectin ligands attenuated lung metastasis in *Fuc-TVII*$^{-/-}$ mice. Reciprocal bone marrow reconstitution experiments showed that endogenous selectin

ligands expressed on hematopoietic but not on stromal cells promote metastasis. This is in agreement with data demonstrating that selectin ligands on leukocytes are required for the recruitment of leukocytes to the inflammatory site.[39] The analysis of leukocyte populations associated with tumor cells in the lungs from *Fuc-TVII*$^{-/-}$ and C57BL/6 mice revealed the contribution of monocytes and granulocytes to this process. Inflammatory monocytes recruited to metastasizing breast and colon tumor cells assist in tumor cell extravasation and metastasis.[12,40] Lungs devoid of endogenous selectin ligands displayed reduced recruitment and association of monocytes with tumor cells. In parallel, adoptively transferred monocytes with intact selectin ligands restored metastasis in *Fuc-TVII*$^{-/-}$ mice, suggesting that endogenous selectin ligands are necessary for the capture of monocytes at the metastatic site, and are therefore required for metastasis.

Tumor cells induce vascular permeability and recruit monocytes in a CCL2-CCR2-dependent manner.[40] Impaired selectin ligand synthesis had no effect on the ability of tumor cells to induce vascular permeability, corroborating our previous findings that tumor cell-derived CCL2 suffices for activation of endothelial cells. Activated endothelial cells also secrete cytokines and chemokines producing a local gradient to attract circulating monocytes.[41] Thus, a comparable chemotactic gradient can be expected at site of tumor cell arrest in C57BL/6 and in *Fuc-TVII*$^{-/-}$ mice. However, reduced association of tumor cells with monocytes provides evidence that monocytes lacking endogenous selectin ligands cannot be captured at the metastatic site. Ly6C^{hi} monocytes express high levels of PSGL-1, which is the ligand for E-, P- and L-selectin.[42] Recruitment of Ly6C^{hi} monocytes to atherosclerotic lesions is PSGL-1 dependent. Accordingly, inhibition of PSGL-1 leads to reduced monocyte accumulation and smaller atherosclerotic plaques.[42] Besides the involvement of Ly6C^{hi} monocytes in the development of atherosclerosis, Ly6C^{hi} monocytes were shown to facilitate metastasis.[12,40] These data support our hypothesis that endogenous selectin ligands are important for the capture of inflammatory monocytes at the metastatic site through interactions with endothelial E-and/or P-selectins. Another possibility is that inflammatory monocytes are recruited to metastatic sites through the process of secondary capture. This

process was observed previously in atherosclerotic lesions where neutrophils were permanently recruited via already arrested neutrophils on the endothelium.[43-45] Secondary capture is mediated exclusively by L-selectin.[44] Since monocytes express L-selectin and L-selectin ligands, they could also interact with rolling or adherent neutrophils. Whether secondary capture-dependent leukocyte recruitment contributes to metastasis remains to be explored.

4.2. Endogenous selectin ligands activate monocytes through outside-in signaling

The chemokine expression profile of lung homogenates from C57BL/6 and *Fuc-TVII*$^{-/-}$ mice after tumor cell injection revealed reduced CCL2 levels in lungs devoid of endogenous selectin ligands. Tumor cells, monocytes, neutrophils and endothelial cells produce CCL2.[46,47] Similar vascular permeability in Fuc-TVII deficient, tumor cell-bearing lungs, indicates that defective selectin ligand synthesis might lead to reduced CCL2 production only in the host cells. It is likely that impaired selectin ligand-mediated outside-in signaling affects CCL2 production in leukocytes. Selectins trigger signal transduction in both selectin-expressing and ligand-expressing cells.[48-50] Selectin ligand-mediated activation of leukocytes has been reported previously.[51,52] Adhesion of myeloid cells to platelets via P-selectin-PSGL-1 interactions induced translational amplification of urokinase plaminogen activator receptor (uPAR) in myeloid cells.[53] Interaction of monocytes with immobilized P-selectin, induced nuclear translocation of NF-kB and led to increased secretion of cytokines like TNFα and CCL2.[54] Recruitment of inflammatory monocytes and their subsequent capture and activation at the metastatic site might explain the up-regulation of CCL2 in C57BL/6 lungs. CCL2 production by activated monocytes could enhance the local CCL2 gradient and in addition to tumor cell-derived CCL2 contribute to the permanent recruitment of inflammatory monocytes that are required for efficient tumor cell extravasation. The ability of tumor cells to elevate CCL2 expression in host cells has been reported previously. [29] Prostate tumor cells up-regulated CCL2 expression in osteoblasts (3-fold) and endothelial cells (2-fold) via secretion of parathyroid hormone-related protein (PTHrP).[29] Metastasizing breast cancer cells triggered

an inflammatory stress response in osteoclasts, including production of CCL2.[55] However, the fact that endogenous selectin ligands might also trigger CCL2 expression at the metastatic site is rather novel.

Based on our work we propose the following model describing the involvement of endogenous selectin ligands in metastasis (Figure 7). After vascular arrest, CCL2-expressing tumor cells produce a local chemokine gradient that is required for the recruitment of $CCR2^{+}$ monocytes. Simultaneously or subsequently, tumor-cells induce vascular permeability via endothelial CCR2. Selectin-selectin ligand interactions mediate adhesion of recruited monocytes at the metastatic site which, following activation, promote tumor cell extravasation and metastasis.

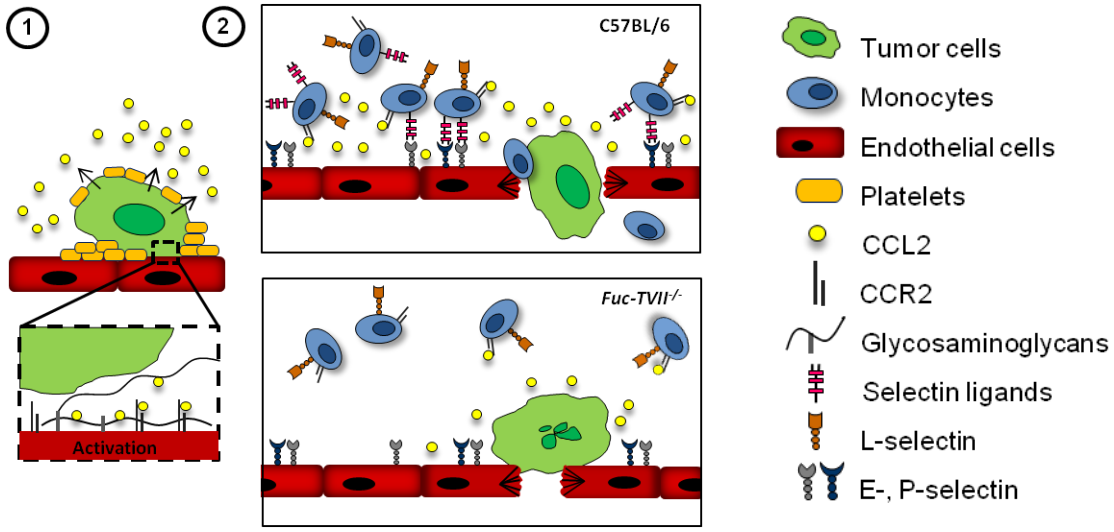


Figure 7: Selectin ligand-mediated capture of monocytes at sites of tumor cell arrest.
This schematic model illustrates how endogenous selectin ligands expressed on monocytes contribute to metastasis. Adhesion of tumor cell emboli to the vasculature triggers the activation of endothelial cells and the production of the local CCL2 gradient (step1). CCL2 recruits inflammatory monocytes and induces vascular permeability through CCL2-CCR2 signaling (step 2). Selectin ligands expressed on monocytes interact with selectins displayed on activated endothelium ensuring the adhesion of recruited monocytes at the metastatic site where they promote tumor cell extravasation. The absence of endogenous selectin ligands on monocytes prevents their adherence at the site of tumor cell arrest, resulting in reduced tumor cell survival and metastasis. In addition, lack of selectin ligands is linked to reduced CCL2 production, suggesting that selectin ligand-mediated outside-in signaling triggers monocyte activation and provides monocytes with metastasis promoting features.

4.3. Neutrophils - additional regulators of metastasis?

Leukocytosis and high infiltration of neutrophils into the lungs raise the question whether neutrophils contribute to attenuated metastasis in *Fuc-TVII*$^{-/-}$ mice. Neutrophils were shown to regulate lung metastasis through interaction with, and anchoring of circulating melanoma cells to the endothelium.[56] Neutrophil-melanoma cell interactions promote tumor cell retention within the lung circulation, enhancing extravasation under flow conditions and subsequent metastasis development.[56] In contrast, in our model, high infiltration of neutrophils did not promote metastasis. Instead, high association of tumor cells with neutrophils correlated with reduced tumor cell retention within the lung and abolished metastasis. This data indicates that different tumor cells use diverse mechanisms and exploit different cell types in their microenvironment in order to metastasize. Depending on their polarization status neutrophils exert dual activities not only in the primary tumor but apparently also at the metastatic site.[57,58]

Several studies report that reduced recruitment of monocytes goes in hand with increased association of tumor cells with neutrophils.[18,34,40] Thus, monocytes ability to inhibit neutrophil infiltration has been suggested.[34] This plasticity in leukocyte recruitment corresponds with our findings and explains enhanced neutrophil association in the case of impaired capture of monocytes to the metastatic site. Therefore, we hypothesized that tumor cells that are not extravasated within 24 hours as a consequence of reduced monocyte capture are recognized by neutrophils and are eliminated by acute neutrophil response. Neutrophils secrete cytotoxic mediators that have been shown to induce apoptosis and lysis of different tumor cells *in vitro* and *in vivo.*[58] Indeed, increased numbers of apoptotic cells were detected in lungs of *Fuc-TVII*$^{-/-}$ mice compared to C57BL/6 mice 24 hours after tumor cell injection. Taken together, these data suggest, that the absence of endogenous selectin ligands results in reduced monocyte capture, subsequent neutrophil recruitment, elimination of non-extravasated tumor cells and reduced metastasis. The phenomenon of observed neutrophil recruitment to the lungs within 24 hours is unclear. Based on the 5-fold increase in neutrophil

infiltration we can exclude that recruitment of neutrophils occurs in a selectin ligand-dependent manner. This is in agreement with previous data showing that neutrophils were able to infiltrate some organs including lungs by a selectin-selectin ligand independent mechanism.[59-62] For instance, elevated levels of GM-CSF and IL7 induced granulopoiesis in lungs of leukocyte adhesion molecule-deficient mice.[63] Another study reported that endothelial CXCR2 mediated the migration of polymorphonuclear leukocytes into the lung in a murine model of acute lung injury.[19] Since mRNA levels of CXCR2 ligands CXCL1, CXCL2 and CXCL5 were not detectable in lungs of *Fuc-TVII*$^{-/-}$ mice 24 hours after tumor cell injection, the contribution of CXCR2 to neutrophil recruitment in our model is not significant.

Of note, cytokine receptors are frequently glycosylated. It has been reported, that fucosylation of the core protein is required for the activation of TGFβ receptor.[64] Similarly, interaction between chemokine IL-8 and its receptor CXCR2 is fucose-dependent.[65] Thus, formation of the chemotactic gradient at the metastatic site might be affected due to impaired fucosylation of the cytokine receptors that trigger inflammation. Since CCR2 is a glycoprotein[66], it is also possible that impaired fucosylation of the core protein could influence CCR2-mediated outside-in signal transduction.

5. Synopsis and further directions

This study describes the molecular mechanism of CCL2-mediated metastatic spread. CCL2 expressing-tumor cells are able to interact with endothelium through the CCR2 receptor which leads to increased vascular permeability due to activation of the Jak2 and p38 pathways. Tumor-derived CCL2 induces the recruitment of $CCR2^{+}$ monocytes that facilitate tumor cell extravasation through the “leaky” endothelium. However, the recruitment of monocytes is not sufficient to promote metastasis. Monocytes further require endogenous selectin ligands to enable their capture and subsequent activation at the site of tumor cell arrest, thus acquiring their pro-metastatic activities. As such, approaches targeting either the

CCL2-CCR2 axis or endogenous selectin ligand interactions unveil new strategies in cancer therapy.

These findings raise several questions:

- How exactly are CCL2-mediated events orchestrated during tumor cell extravasation?
- How can endogenous selectin ligands mediate CCL2 expression?
- Do other cancer cells also use chemokine-chemokine receptor interactions to accomplish tumor cell extravasation?

Consequently, further investigations will provide detailed insights in the role of endogenous selectin ligands, chemokines and chemokine receptors in metastasis.

6. References

1. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2009;9:239-252.
2. Balkwill F. Chemokine biology in cancer. Semin Immunol. 2003;15:49-55.
3. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer. 2006;42:717-727.
4. O'Hayre M, Salanga CL, Handel TM, Allen SJ. Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. Biochem J. 2008;409:635-649.
5. Zhang J, Patel L, Pienta KJ. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. Cytokine Growth Factor Rev. 2009;21:41-48.
6. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001;410:50-56.
7. Walser TC, Rifat S, Ma X, Kundu N, Ward C, Goloubeva O, Johnson MG, Medina JC, Collins TL, Fulton AM. Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. Cancer Res. 2006;66:7701-7707.
8. Vande Broek I, Asosingh K, Vanderkerken K, Straetmans N, Van Camp B, Van Riet I. Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemotactic proteins MCP-1, -2 and -3. Br J Cancer. 2003;88:855-862.
9. Lu Y, Chen Q, Corey E, Xie W, Fan J, Mizokami A, Zhang J. Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone. Clin Exp Metastasis. 2009;26:161-169.
10. Huang B, Lei Z, Zhao J, Gong W, Liu J, Chen Z, Liu Y, Li D, Yuan Y, Zhang GM, Feng ZH. CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. Cancer Lett. 2007;252:86-92.
11. Mizutani K, Sud S, McGregor NA, Martinovski G, Rice BT, Craig MJ, Varsos ZS, Roca H, Pienta KJ. The chemokine CCL2 increases prostate tumor growth and bone metastasis through macrophage and osteoclast recruitment. Neoplasia. 2009;11:1235-1242.
12. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA, Pollard JW. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature. 2011;475:222-225.
13. Said N, Sanchez-Carbayo M, Smith SC, Theodorescu D. RhoGDI2 suppresses lung metastasis in mice by reducing tumor versican expression and macrophage infiltration. J Clin Invest. 2012;122:1503-1518.
14. Keane MP, Belperio JA, Xue YY, Burdick MD, Strieter RM. Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer. J Immunol. 2004;172:2853-2860.
15. Zijlmans HJ, Fleuren GJ, Baelde HJ, Eilers PH, Kenter GG, Gorter A. The absence of CCL2 expression in cervical carcinoma is associated with increased survival and loss of heterozygosity at 17q11.2. J Pathol. 2006;208:507-517.
16. Yoshidome H, Kohno H, Shida T, Kimura F, Shimizu H, Ohtsuka M, Nakatani Y, Miyazaki M. Significance of monocyte chemoattractant protein-1 in angiogenesis and survival in colorectal liver metastases. Int J Oncol. 2009;34:923-930.
17. Soria G, Ofri-Shahak M, Haas I, Yaal-Hahoshen N, Leider-Trejo L, Leibovich-Rivkin T, Weitzenfeld P, Meshel T, Shabtai E, Gutman M, Ben-Baruch A. Inflammatory mediators in breast cancer: coordinated expression of TNFalpha & IL-1beta with CCL2 & CCL5 and effects on epithelial-to-mesenchymal transition. BMC Cancer. 2011;11:130.
18. Sawanobori Y, Ueha S, Kurachi M, Shimaoka T, Talmadge JE, Abe J, Shono Y, Kitabatake M, Kakimi K, Mukaida N, Matsushima K. Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. Blood. 2008;111:5457-5466.
19. Reutershan J, Morris MA, Burcin TL, Smith DF, Chang D, Saprito MS, Ley K. Critical role of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. J Clin Invest. 2006;116:695-702.

20. Huang Y, Song N, Ding Y, Yuan S, Li X, Cai H, Shi H, Luo Y. Pulmonary vascular destabilization in the premetastatic phase facilitates lung metastasis. Cancer Res. 2009;69:7529-7537.
21. McIntyre TM, Prescott SM, Weyrich AS, Zimmerman GA. Cell-cell interactions: leukocyte-endothelial interactions. Curr Opin Hematol. 2003;10:150-158.
22. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007;7:678-689.
23. Cara DC, Kaur J, Forster M, McCafferty DM, Kubes P. Role of p38 mitogen-activated protein kinase in chemokine-induced emigration and chemotaxis in vivo. J Immunol. 2001;167:6552-6558.
24. Sanz-Moreno V, Gaggioli C, Yeo M, Albrengues J, Wallberg F, Viros A, Hooper S, Mitter R, Feral CC, Cook M, Larkin J, Marais R, Meneguzzi G, Sahai E, Marshall CJ. ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. Cancer Cell. 2011;20:229-245.
25. Tremblay PL, Auger FA, Huot J. Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of p38 and ERK MAP kinases. Oncogene. 2006;25:6563-6573.
26. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer. 2009;9:798-809.
27. Agrawal S, Gollapudi S, Su H, Gupta S. Leptin activates human B cells to secrete TNF-alpha, IL-6, and IL-10 via JAK2/STAT3 and p38MAPK/ERK1/2 signaling pathway. J Clin Immunol. 2011;31:472-478.
28. Loberg RD, Day LL, Harwood J, Ying C, St John LN, Giles R, Neeley CK, Pienta KJ. CCL2 is a potent regulator of prostate cancer cell migration and proliferation. Neoplasia. 2006;8:578-586.
29. Li X, Loberg R, Liao J, Ying C, Snyder LA, Pienta KJ, McCauley LK. A destructive cascade mediated by CCL2 facilitates prostate cancer growth in bone. Cancer Res. 2009;69:1685-1692.
30. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood. 2000;96:34-40.
31. Leuschner F, Dutta P, Gorbatov R, Novobrantseva TI, Donahoe JS, Courties G, Lee KM, Kim JI, Markmann JF, Marinelli B, Panizzi P, Lee WW, Iwamoto Y, Milstein S, Epstein-Barash H, Cantley W, Wong J, Cortez-Retamozo V, Newton A, Love K, Libby P, Pittet MJ, Swirski FK, Koteliansky V, Langer R, Weissleder R, Anderson DG, Nahrendorf M. Therapeutic siRNA silencing in inflammatory monocytes in mice. Nat Biotechnol. 2011;29:1005-1010.
32. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860-867.
33. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002;23:549-555.
34. Pahler JC, Tazzyman S, Erez N, Chen YY, Murdoch C, Nozawa H, Lewis CE, Hanahan D. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. Neoplasia. 2008;10:329-340.
35. Kannagi R. Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. Glycoconj J. 1997;14:577-584.
36. Borsig L, Stevenson JL, Varki A. Heparin in Cancer: Role of Selectin Interactions. In: Khorana AA, Francis CW, eds. Cancer-Associated Thrombosis New York: Informa Healthcare; 2007:97-113.
37. Witz IP. The selectin-selectin ligand axis in tumor progression. Cancer Metastasis Rev. 2008;27:19-30.
38. Laubli H, Stevenson JL, Varki A, Varki NM, Borsig L. L-selectin facilitation of metastasis involves temporal induction of Fut7-dependent ligands at sites of tumor cell arrest. Cancer Res. 2006;66:1536-1542.
39. Sperandio M, Smith ML, Forlow SB, Olson TS, Xia L, McEver RP, Ley K. P-selectin glycoprotein ligand-1 mediates L-selectin-dependent leukocyte rolling in venules. J Exp Med. 2003;197:1355-1363.
40. Wolf MJ, Hoos A, Bauer J, Boettcher S, Knust M, Weber A, Simonavicius N, Schneider C, Lang M, Sturzl M, Croner RS, Konrad A, Manz MG, Moch H, Aguzzi A, van Loo G, Pasparakis M, Prinz M, Borsig L, Heikenwalder M. Endothelial CCR2 Signaling Induced by Colon Carcinoma Cells Enables Extravasation via the JAK2-Stat5 and p38MAPK Pathway. Cancer Cell. 2012;22:91-105.

41. Laubli H, Spanaus KS, Borsig L. Selectin-mediated activation of endothelial cells induces expression of CCL5 and promotes metastasis through recruitment of monocytes. Blood. 2009;114:4583-4591.
42. An G, Wang H, Tang R, Yago T, McDaniel JM, McGee S, Huo Y, Xia L. P-selectin glycoprotein ligand-1 is highly expressed on Ly-6Chi monocytes and a major determinant for Ly-6Chi monocyte recruitment to sites of atherosclerosis in mice. Circulation. 2008;117:3227-3237.
43. Bargatze RF, Kurk S, Butcher EC, Jutila MA. Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. J Exp Med. 1994;180:1785-1792.
44. Alon R, Fuhlbrigge RC, Finger EB, Springer TA. Interactions through L-selectin between leukocytes and adherent leukocytes nucleate rolling adhesions on selectins and VCAM-1 in shear flow. J Cell Biol. 1996;135:849-865.
45. Eriksson EE, Xie X, Werr J, Thoren P, Lindbom L. Importance of primary capture and L-selectin-dependent secondary capture in leukocyte accumulation in inflammation and atherosclerosis in vivo. J Exp Med. 2001;194:205-218.
46. Yadav A, Saini V, Arora S. MCP-1: chemoattractant with a role beyond immunity: a review. Clin Chim Acta. 2010;411:1570-1579.
47. Fridlender ZG, Sun J, Mishalian I, Singhal S, Cheng G, Kapoor V, Horng W, Fridlender G, Bayuh R, Worthen GS, Albelda SM. Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. PLoS One. 2012;7:e31524.
48. Lorenzon P, Vecile E, Nardon E, Ferrero E, Harlan JM, Tedesco F, Dobrina A. Endothelial cell E- and P-selectin and vascular cell adhesion molecule-1 function as signaling receptors. J Cell Biol. 1998;142:1381-1391.
49. Yoshida M, Szente BE, Kiely JM, Rosenzweig A, Gimbrone MA, Jr. Phosphorylation of the cytoplasmic domain of E-selectin is regulated during leukocyte-endothelial adhesion. J Immunol. 1998;161:933-941.
50. Hu Y, Szente B, Kiely JM, Gimbrone MA, Jr. Molecular events in transmembrane signaling via E-selectin. SHP2 association, adaptor protein complex formation and ERK1/2 activation. J Biol Chem. 2001;276:48549-48553.
51. Crockett-Torabi E. Selectins and mechanisms of signal transduction. J Leukoc Biol. 1998;63:1-14.
52. Urzainqui A, Serrador JM, Viedma F, Yanez-Mo M, Rodriguez A, Corbi AL, Alonso-Lebrero JL, Luque A, Deckert M, Vazquez J, Sanchez-Madrid F. ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. Immunity. 2002;17:401-412.
53. Mahoney TS, Weyrich AS, Dixon DA, McIntyre T, Prescott SM, Zimmerman GA. Cell adhesion regulates gene expression at translational checkpoints in human myeloid leukocytes. Proc Natl Acad Sci U S A. 2001;98:10284-10289.
54. Weyrich AS, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation. J Clin Invest. 1995;95:2297-2303.
55. Kinder M, Chislock E, Bussard KM, Shuman L, Mastro AM. Metastatic breast cancer induces an osteoblast inflammatory response. Exp Cell Res. 2008;314:173-183.
56. Huh SJ, Liang S, Sharma A, Dong C, Robertson GP. Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. Cancer Res. 2010;70:6071-6082.
57. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell. 2009;16:183-194.
58. Piccard H, Muschel RJ, Opdenakker G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. Crit Rev Oncol Hematol. 2011;82:296-309.
59. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. Cell. 1993;74:541-554.
60. Bullard DC, Qin L, Lorenzo I, Quinlin WM, Doyle NA, Bosse R, Vestweber D, Doerschuk CM, Beaudet AL. P-selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the

peritoneum is completely absent but is normal into pulmonary alveoli. J Clin Invest. 1995;95:1782-1788.
61. Yang J, Hirata T, Croce K, Merrill-Skoloff G, Tchernychev B, Williams E, Flaumenhaft R, Furie BC, Furie B. Targeted gene disruption demonstrates that P-selectin glycoprotein ligand 1 (PSGL-1) is required for P-selectin-mediated but not E-selectin-mediated neutrophil rolling and migration. J Exp Med. 1999;190:1769-1782.
62. Xia L, Sperandio M, Yago T, McDaniel JM, Cummings RD, Pearson-White S, Ley K, McEver RP. P-selectin glycoprotein ligand-1-deficient mice have impaired leukocyte tethering to E-selectin under flow. J Clin Invest. 2002;109:939-950.
63. Forlow SB, Schurr JR, Kolls JK, Bagby GJ, Schwarzenberger PO, Ley K. Increased granulopoiesis through interleukin-17 and granulocyte colony-stimulating factor in leukocyte adhesion molecule-deficient mice. Blood. 2001;98:3309-3314.
64. Wang X, Inoue S, Gu J, Miyoshi E, Noda K, Li W, Mizuno-Horikawa Y, Nakano M, Asahi M, Takahashi M, Uozumi N, Ihara S, Lee SH, Ikeda Y, Yamaguchi Y, Aze Y, Tomiyama Y, Fujii J, Suzuki K, Kondo A, Shapiro SD, Lopez-Otin C, Kuwaki T, Okabe M, Honke K, Taniguchi N. Dysregulation of TGF-beta1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. Proc Natl Acad Sci U S A. 2005;102:15791-15796.
65. Wu LH, Shi BZ, Zhao QL, Wu XZ. Fucosylated glycan inhibition of human hepatocellular carcinoma cell migration through binding to chemokine receptors. Glycobiology. 2009;20:215-223.
66. Preobrazhensky AA, Dragan S, Kawano T, Gavrilin MA, Gulina IV, Chakravarty L, Kolattukudy PE. Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular N-terminal region. J Immunol. 2000;165:5295-5303.

ACKNOWLEDGMENTS

First of all, I would like to thank PD Dr. Lubor Borsig for giving me the opportunity to do research in his lab and for providing me with interesting projects. Thanks a lot for the competent supervision, intensive scientific discussions that led to many interesting ideas and useful experiments, constructive criticism and great support with the animal work during the four years of my thesis.

I sincerely thank the members of my Ph.D. committee, Professors Anne Müller, Reto Schwendener and Thierry Hennet for their time, discussions, suggestions and critical questions on my projects. Special thanks to Prof. Müller for agreeing to be my "Dissertations Leiterin".

I am very grateful to all the people involved in the CCR2 project. I thank Prof. Dr. Mathias Heikenwälder, Dr. Monika Wolf and Judith Bauer for successful collaborations, valuable input and technical support. Special thanks to Dr. Monika Wolf for helping us with the irradiation of mice for the Fuc-TVII project, and for sharing and exchanging knowledge and resources. I am thankful to technical assistants (Ruth and Daniel) from Technical University Munich for performing paraffin blocks and staining.

Further I would like to thank people on the L-and K floor for technical support and sharing antibodies, primers and chemical reagents. Thanks to Prof. Devuyst and Huguette Debaix for helping out with the RT-PCR machine. Big thanks to people from the Center for Microscopy and Image Analysis (Caroline, Urs, José) for providing advice and by assisting in adjustments for good quality pictures. Next, I am very grateful to our animal care takers (Daniel, Sabrina, Matthew) for keeping my mice in good condition. Many thanks to Esther for never giving up while keeping track of our bills.

I am very grateful to Stephan Baumann for providing great support with computer and computer-related business.
Huge thanks to Dr. Kelvin Luther for correcting my thesis and for valuable suggestions and comments during my progress reports.

Further I would like to thank:
Andrea Fuhrer for sharing her experience and reagents; Darya Protsyuk for helping me to complete the figure for the paper and continuing work on the Fuc-TVII project. I am sure, it will be worth the price. Thanks to Marco Roblek und Irina Häuselmann for technical support in the lab and valuable input during our board meetings; Jesús just for being Jesús.

I also thank all former and present members of the Borsig and Hennet groups for the warm working atmosphere, many apéros, flat worming parties, extra beers, Jumbo Schnitzels and so on. Special thanks to Anna and Adrienne for supplying us with extra portions of sugar in the form of tasty cakes and cup cakes.

Thanks to the PhD Cancer Program for creating a competitive, international working environment, organizing courses, PhD retreats and other leisure activities. It was fun to be involved.

Special thanks to my dear friends Katya Kurakevich, Carole Oertli and Marko Roblek for being there in good and bad times, for great support during FACS and Microscope sessions, for our “coffee breaks” and funny video evenings.

To my dear family, relatives and friends, I appreciate so much your constant support and encouragement during tough times. Thanks for giving me the balance and other life perspectives out of the lab and for unconditional love throughout my life.

I am also very grateful to the Dupont and Rochat families. Thanks for an affectionate welcome in Switzerland and into your family. Thanks for great sailing tours and exciting family weekends.

Finally, my special recognition goes out to my sweetheart. Thank you so much for being always there, for your amazing cooking and housekeeping, for your “crazy moments” to cheer me up. This achievement would not have been possible without your support.

CURRICULUM VITAE

Personal data

Name:	Alexandra Hoos
E-mail address:	Alexandra.Hoos@uzh.ch
Date and place of birth:	November 26, 1981 in Kazakhstan/Astana
Nationality:	German

Education

04/2009 - 04/2013	Ph.D. thesis at the Institute of Physiology, University of Zurich; in the group of PD Dr. Lubor Borsig; Cancer Biology Ph.D. program "Selectin-driven leukocyte recruitment and chemokines facilitate metastasis"
08/2007 - 12/2008	Master thesis at the Institute of Zoology, Department of Cell and Matrix Biology, University of Mainz, Germany; in the group of Prof. Dr. W. Stöcker "Identification and characterization of the homologous interstitial human serinproteases from the Zebra danio (*Danio rerio)*"
09/2006 - 07/2007	Exchange program at the University of Cardiff, Wales; in the department of Molecular Biosciences
09/2002 - 09/2006	Studies in Biology at the University of Mainz, Germany; with the focus on Molecular Zoology, Genetics and Physiological Chemistry
09/2001 - 03/2002	Grammar School "Staatliches Eifel Gymnasium" in Neuerburg, Germany; certificate of complete general secondary education
08/1999 - 06/2001	Grammar School "Thomas Morus Gymnasium" in Daun, Germany

Conferences & retreats

20 - 25.05.2012	Keystone Symposia/ Dublin/ The Role of Inflammation during Carcinogenesis/ Poster
07 - 09.03.2012	Cancer Biology Ph.D. student Retreat/ Interlaken/ Poster
06 - 07.02.2012	USGEB Annual Meeting/ Lausanne/ Talk
25 - 27.03.2011	Joined Ph.D. student Retreat with EPFL/ Les Diablerets/ Talk
25 - 27.10.2010	Cancer Biology Ph.D. student Retreat/ Wilderswil/ Talk

Awarded grants

08/2006	ERASMUS
06/2012	Cancer Biology Ph.D. Program Travel Grant

Publications

Alexandra Hoos, Lubor Borsig. (2013) Selectin ligands on leukocytes are required for monocyte recruitment and adhesion at metastatic sites. Submitted

Alexandra Hoos*, Monika Julia Wolf*, Judith Bauer, Steffen Boettcher Markus Knust, Achim Weber, Nicole Simonavicius, Christoph Schneider, Matthias Lang, Michael Stürzl, Roland S. Croner, Andreas Konrad, Markus G. Manz, Holger Moch, Adriano Aguzzi, Geert van Loo, Manolis Pasparakis, Marco Prinz, Lubor Borsig*, Mathias Heikenwalder*. (2012) *Endothelial CCR2 Signaling Induced by Colon Carcinoma Cells Enables Extravasation via the JAK2-Stat5 and p38MAPK Pathway*. Cancer Cell 22: 91–105

Alexandra Hoos, Monika Julia Wolf, Judith Bauer, Lubor Borsig, Mathias Heikenwalder. (2012) *Endothelial chemokine receptors as facilitators of tumor cell extravasation?* Oncotarget 3: 919-920

Björn Müller-Edenborn, Birgit Roth-Z'graggen, Kamila Bartnicka, Alain Borgeat, **Alexandra Hoos**, Lubor Borsig, Beatrice Beck-Schimmer. (2012) *Volatile Anesthetics Reduce Invasion of Colorectal Cancer Cells through Down-regulation of Matrix Metalloproteinase-9.* Anesthesiology: 293–301

* these authors contributed equally

APPENDIX

Endothelial chemokine receptors as facilitators of tumor cell extravasation?

Article published in Journal of Oncotarget, 2012

Authors: Alexandra Hoos, Monika Julia Wolf, Judith Bauer, Lubor Borsig and Mathias Heikenwalder

Contributions: I and M. J. W. wrote the manuscript. L.B. and M. H. read and corrected the manuscript.

www.impactjournals.com/oncotarget/ Oncotarget, September, Vol.3, No 9

Endothelial chemokine receptors as facilitators of tumor cell extravasation?

Alexandra Hoos, Monika J. Wolf, Judith Bauer, Lubor Borsig and Mathias Heikenwalder

Metastasis is a multistep process characterized by the ability of tumor cells to "communicate" and to interact with their microenvironment to establish tumors in distant organs. A significant proportion of the metastatic microenvironment consists of leukocytes, mostly of the innate immune system, contributing to tumor invasion and outgrowth. Chemokines are instrumental for recruiting immune cells thereby enabling efficient metastasis [1]. Still, the exact mechanisms of chemokine action in the metastatic process have remained elusive. In the context of cancer, chemokines were shown to affect leukocyte recruitment, tumor cell proliferation, invasion, angiogenesis and cancer progression [2]. In addition, tissue-specific metastasis of certain cancers was shown to depend on specific chemokine-chemokine receptor interactions. For instance, the expression of the chemokine receptor CXCR4 on breast cancer cells supports metastasis to secondary sites expressing CXCL12 [3]. Chemokine-driven leukocyte recruitment in tumors is linked to the major attractors of monocytic cells, namely CCL2 and CCL5 [4, 5]. Elevated levels of CCL2 correlated with poor prognosis due to metastasis in a variety of cancer patients e.g. suffering from breast and prostate cancer. In a recent study we detected elevated levels of CCL2 in biopsies from metastatic colon cancer patients (UICC stage IV) [6].

Still, the different roles of CCL2 and CCL5 during metastasis are just at the beginning to be defined. In the tumor microenvironment, stromal cells, infiltrating leukocytes and tumor cells themselves were identified as sources of chemokines. During the initial phase of metastasis, local activation of endothelia by tumor cells induced endothelial CCL5 expression, resulting in monocyte recruitment and promotion of metastasis [7]. Recently, CCL2-mediated recruitment of monocytes has been identified as the major factor facilitating breast cancer metastasis to the lung [8], while the exact role of monocytes in this process remained unanswered. We showed that monocytes enable tumor cell transmigration through the endothelium [6]. Using mouse models we demonstrated that expression of CCL2 by colon cancer cells not only attracts pro-inflammatory monocytes but also directly signals towards $CCR2^+$ endothelial cells to enable tumor cell extravasation. Importantly, tumor cells were unable to efficiently transmigrate through CCR2-deficient endothelial cells, indicating the presence of CCR2 on the endothelium is a crucial factor for CCL2-mediated metastasis. Activation of CCR2 on endothelial cells through the JAK2-Stat5 and p38MAPK pathway caused increased vascular permeability and enhanced tumor cell transmigration *in vivo* and *in vitro*. Moreover, blocking of signaling pathways downstream of CCR2 significantly reduced metastasis, supporting the notion that endothelial CCR2 "licenses" colon cancer extravasation. Consequently, our findings define an additional role for chemokines in enabling metastasis that goes beyond the attraction of inflammatory leukocytes.

We confirmed that metastasis of another tumor cell line, Lewis lung carcinoma (3LL), is also dependent on CCL2 expression. In contrast, the melanoma cell line B16-BL6 could extravasate and metastasize independently of endothelial CCR2 expression. Interestingly, this cell line expresses only little CCL2. Our findings raise several questions:

(1) Do other cancer cells also use chemokine-chemokine receptor interactions to accomplish tumor cell extravasation?

(2) Can we use chemokine expression analysis in primary tumors of patients to predict increase risk for metastasis?

(3) What other chemokine-chemokine receptor pairs exist, having the same or other biological function as CCL2-CCR2?

(4) What do we know about the cellular and spatiotemporal denominators of CCL2-induced tumor cell extravasation?

Consequently, further experiments will show how general our recently reported findings are and whether other chemokine receptors on endothelial cells exert similar functions. We hypothesize that other functions of tumor cell-derived chemokines will be identified in the future, surpassing their known role as immune cell attractors, as the currently identified contribution to induced vascular permeability.

Alexandra Hoos: Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland
Monika J. Wolf: Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland
Judith Bauer: Institute of Virology, Technische Universität München/Helmholtz Zentrum Munich, Munich, Germany

Lubor Borsig: Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland
Mathias Heikenwalder: Institute of Virology, Technische Universität München/Helmholtz Zentrum Munich, Munich, Germany

***Correspondence**: Mathias Heikenwalder, **email** heikenwaelder@helmholtz-muenchen.de*

***Received**: September 17, 2012;*

***Published**: September 19, 2012;*

REFERENCES

1. Zlotnik A, Burkhardt AM, Homey B. Nat Rev Immunol. 2011; 11:597-606.
2. Allavena P, Germano G, Marchesi F, Mantovani A. Exp Cell Res. 2011; 317:664-673.
3. Muller A, Homey B, Soto H et al. Nature. 2001; 410:50-56.
4. Soria G, Ben-Baruch A. Cancer Lett. 2008; 267:271-285.
5. Balkwill F. Nat Rev Cancer. 2004; 4:540-550.
6. Wolf MJ, Hoos A, Bauer J et al. Cancer Cell. 2012; 22:91-105.
7. Läubli H, Spanaus KS, Borsig L. Blood. 2009; 114:4583-4591.
8. Qian BZ, Li J, Zhang H et al. Nature. 2011; 475:222-225.

Volatile Anesthetics Reduce Invasion of Colorectal Cancer Cells through Down-regulation of Matrix Metalloproteinase-9

Article published in Journal of Anesthesiology, 2012

Authors: Björn Müller-Edenborn, Birgit Roth-Z'graggen, Kamila Bartnicka, Alain Borgeat, Alexandra Hoos, Lubor Borsig, Beatrice Beck-Schimmer.

Contributions: I provided technical knowledge and cells for the experiments shown in figure 6.

Volatile Anesthetics Reduce Invasion of Colorectal Cancer Cells through Down-regulation of Matrix Metalloproteinase-9

Björn Müller-Edenborn, M.D.,* Birgit Roth-Z'graggen, Ph.D.,† Kamila Bartnicka, M.Sc.,‡ Alain Borgeat, M.D.,§ Alexandra Hoos, M.Sc.,‡ Lubor Borsig, Ph.D.,‖ Beatrice Beck-Schimmer, M.D.#

ABSTRACT

Background: Invasion of extracellular matrix is a hallmark of malignant tumors. Clamping maneuvers during cancer surgery reduce blood loss, but trigger reperfusion injury (RI). RI increases cancer recurrence in the reperfused organ through up-regulation of matrix metalloproteinase-9 (MMP-9). Interleukin-8 is an important cytokine in RI promoting accumulation of neutrophils, a major source of MMP-9. Volatile anesthetics were demonstrated to reduce RI. We hypothesized that these anesthetics might attenuate MMP-9 up-regulation and consequently tumor cell invasion in RI.

Methods: Isolated human neutrophils (n = 6) were preconditioned with sevoflurane or desflurane, followed by stimulation with interleukin-8, phorbol myristate acetate, or chemokine CXC-ligand 1 (CXCL1) to differentiate intracellular pathways. MMP-9 release and activity were quantified by enzyme-linked immunosorbent assay and zymography, respectively. CXC-receptor-2 (CXCR2) expression and phosphorylation of extracellular signal-regulated kinases 1/2 were assessed by flow cytometry. The impact of MMP-9 on the invasion of neutrophils and MC-38 colon cancer cells was assessed using Matrigel-coated filters (n = 6).

Results: Preconditioning reduced interleukin-8-induced MMP-9-release by 41% (±13, 5%, sevoflurane) and 40% (±13%, desflurane). This was also evident following stimulation of CXCR2 with CXCL1. No impact on phosphorylation of extracellular signal-regulated kinases 1/2 and MMP-9 release was observed with receptor-independent stimulation of protein kinase C with phorbol myristate acetate. Preconditioning reduced transmigration of neutrophils and MC-38 tumor cells to baseline levels.

Discussion: Volatile anesthetics impair neutrophil MMP-9 release and interfere with pathways downstream of CXCR2, but upstream of protein kinase C. Through down-regulation of MMP-9, volatile anesthetics decrease Matrigel breakdown and reduce subsequent migration of cancer cells *in vitro*.

What We Already Know about This Topic

- Matrix metalloproteinase-9 (MMP-9) facilitates tumor invasion and migration by degrading extracellular matrix
- Neutrophil release of MMP-9 through protein kinase C (PKC) activation is increased by interleukin-8 (IL-8) stimulation during postischemia reperfusion
- Potent volatile anesthetics attenuate reperfusion injury and decrease up-regulation of stimulated neutrophil inflammatory adherence molecules

What This Article Tells Us That Is New

- Volatile anesthetic preconditioning reduced MMP-9 release by IL-8-stimulated human neutrophils *in vitro*
- This effect was mediated downstream of the IL-8 receptor and upstream of PKC
- MMP-9 down-regulation reduced mouse colon carcinoma cell migration across simulated extracellular matrix *in vitro*

SURGICAL resection is the primary treatment for patients suffering from colorectal cancer. For higher stages of disease with tumor spread, surgical removal of isolated liver metastases combined with chemotherapy is common and may be curative in selected patients.[1] The 5-yr survival rate of such patients qualifying for curative liver resection is 40–50%, mostly because of recurrent malignancies within the

* Research Assistant and Resident, # Professor, Institute of Anesthesiology, University Hospital Zurich, Zurich, Switzerland, and Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland. † Research Assistant, ‡ Ph.D. Student, ‖ Senior Scientist, Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich. § Professor, Institute of Anesthesiology, University Hospital Zurich-Balgrist, Zurich, Switzerland.

Received from the Institute of Anesthesiology, University of Zurich and University Hospital Zurich, Zurich, Switzerland. Submitted for publication September 2, 2011. Accepted for publication April 4, 2012. Supported by the Swiss National Research Foundation, Bern, Switzerland (grant no. 320030_141216, to Dr. Beck-Schimmer), the Swiss Society of Anesthesiology and Reanimation, Bern, Switzerland (to Dr. Beck-Schimmer and Dr. Müller-Edenborn), and Baxter Schweiz AG, Volketswil, Switzerland (to Dr. Beck-Schimmer).

Address correspondence to Dr. Beck-Schimmer: Institute of Physiology and Centre for Integrative Human Physiology, Institute of Anesthesiology, University of Zurich Medical School, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. beatrice.beckschimmer@uzh.ch. Anesthesiology 2012; 117:293–301

liver.[1] This poor outcome might be partially influenced by the hepatic resection itself. First, surgical manipulation leads to the dissemination of cancer cells into the blood.[2,3] Second, clamping maneuvers to anticipate large blood losses are known to result in reperfusion injury of the liver,[4,5] which has been shown to increase both the number and the size of liver metastasis in animal models of colorectal cancer.[4,6]

There is a considerable amount of evidence that matrix metalloproteinase-9 (MMP-9), also referred to as gelatinase B, plays an important role at different steps of malignant tumor growth.[7] MMP-9 belongs to a family of zinc-dependent gelatinases that degrades components of the extracellular matrix.[8] Before invading blood or lymph vessels, malignant tumor cells are obliged to cross multiple physical barriers, such as the extracellular matrix.[9] By degrading the extracellular matrix, MMP-9 promotes this process and facilitates tumor invasion and migration.[7] Several other proteinases are also able to degrade components of the extracellular matrix. However, matrix metalloproteinases appear to be of particular importance with specific members being predominant for certain malignant tumors.[8] MMP-9 was demonstrated to have a strong impact on colon cancer.[7,10,11]

Albeit the fact that tumor cells are able to produce MMP-9, the richest source of MMP-9 in the body are neutrophils. Neutrophils produce MMP-9 constitutively at a low basal rate and dramatically increase MMP-9 release upon stimulation with the proinflammatory cytokine interleukin-8 (IL-8).[12] High amounts of IL-8 are released during the reperfusion phase after ischemia and promote the influx of large numbers of neutrophils into the reperfused organ.[13–15] Volatile anesthetics were demonstrated to attenuate reperfusion injury *in vivo*[16–18] and to decrease the up-regulation of inflammatory adherence molecules of stimulated neutrophils *in vitro*.[19–21]

Therefore, we hypothesized that volatile anesthetics not only attenuate up-regulation of cell surface molecules, but also reduce the release of MMP-9, stored in neutrophil granules. This study further aims at investigating if volatile anesthetic-induced decreased levels of MMP-9 are functionally potent enough to impair degradation of extracellular matrix and subsequent invasion of tumor cells.

Materials and Methods

Isolation of Neutrophils

Citrated venous blood was collected after informed consent from volunteers with no history of illness in the past 14 days. Ethical approval was received from the Ethic Committee of the University Hospital Zurich (Zurich, Switzerland). Red blood cells were lysed and neutrophils were isolated by gradient centrifugation as described before.[22] The remaining cell suspension was layered over Ficoll-Histopaque 1077 (Sigma-Aldrich, St. Louis, MO), centrifuged at 1,500 rpm for 30 min, and resuspended at 2×10^6 neutrophils/ml in Ham's F-12 medium supplemented with 0.5% bovine serum albumin (Invitrogen, Carlsbad, CA). All steps were performed at 4°C to prevent artificial activation of neutrophils. Before each experiment, neutrophils were examined under the light microscope for signs of artificial activation such as clumping, which were absent.

Preconditioning with Volatile Anesthetics

Neutrophil cell suspension was seeded in sterile 96-well plates at 50 μl per well (10^5 neutrophils/well) and placed in humified airtight chambers (Oxoid anaerobic jar; Oxoid AG, Basel, Switzerland). Chambers were flushed with an air/5% CO_2-mixture for 5 min that was optionally augmented with 2.2% of sevoflurane (Sevorane®; Abbott AG, Baar, Switzerland, corresponding vaporizer: Sevotec5®; Abbott AG) or 6% of desflurane (Forene®; Baxter, Zurich, Switzerland; corresponding vaporizer: Tec6, Carbamed; Liebefeld, Switzerland). Control cells were exposed to the air/5% CO_2-mixture only. Volatile anesthetic concentrations were measured by the Ohmeda 5330 Agent Monitor (Abbott AG). Chambers were sealed and kept in an incubator (Bioblock, Ittingen, Switzerland) at 37°C for 45min (preconditioning = exposure to volatile anesthetic before stimulation). The concentrations of the volatile anesthetic were again checked at the end of incubation to preclude insufficient sealing.

Stimulation of Neutrophils

Following preconditioning, neutrophils were removed from the chambers and put in an incubator at 37°C flushed with an air/5% CO_2-mixture. Neutrophils were immediately stimulated with 100 nM human IL-8 (recombinant human IL-8, BD Pharmingen, Franklin Lakes, NJ) for 15 min. Neutrophils exposed to air/5% CO_2-mixture during preconditioning followed by stimulation with phosphate-buffered saline served as negative controls. Stimulation was stopped on ice and supernatants were harvested for further analysis.

For experiments on protein kinase C (PKC)-dependent extracellular signal-regulated kinase (ERK) 1/2 phosphorylation and MMP-9 release, neutrophils were preconditioned (see Preconditioning with Volatile Anesthetics), followed by stimulation with 100 nM phorbol myristate acetate (PMA) for 5 min. Stimulation with the chemokine CXC-ligand 1 (CXCL1) at 100 ng/ml for 15 min was used in experiments to trigger through CXC-receptor 2 (CXCR2) MMP-9-release.

Quantification of Total MMP-9 in Supernatant

Concentration of MMP-9 was determined using an enzyme-linked immunosorbent assay (ELISA), according to manufacturer's directions (R&D systems, Minneapolis, MN). This assay measures the 92 kDa pro-MMP-9 and the 82 kDa active MMP-9.

Measurement of Enzymatic Activity of MMP-9

Activity of MMP-9 in supernatants from IL-8- and PMA-stimulated neutrophils was measured using zymography, as described before.[23] Zymography gels containing gelatin as a substrate for MMP-9 were used. MMP-9 activity was indirectly evaluated by quantification of the degraded amount of

gelatin using densitometry. A control of human recombinant MMP-9 (R&D Systems) was run with every gel together with protein molecular marker (SeeBlue2, Invitrogen) to confirm the MMP-9-band. Data were analyzed with ImageJ (Wayne Rasband, Bethesda, MD).

Flow Cytometric Measurement of Neutrophil Viability

Unstimulated neutrophils were exposed to volatile anesthetics (see Preconditioning with Volatile Anesthetics). Cells were harvested and incubated with the cell impermeable DNA-dye 7-aminoactinomycin (7-AAD, BD Biosciences, Franklin Lakes, NJ) for 20 min at room temperature and analyzed by flow cytometry (FACS Canto II, BD Biosciences). Viable cells were defined as staining negative for 7-AAD.

Flow Cytometric Measurement of PKC-dependent ERK1/2 Phosphorylation

Following preconditioning (see Preconditioning with Volatile Anesthetics), neutrophils were stimulated with 100 nM PMA for 5 min at 37°C. Staining of phosphorylated ERK1/2 was performed as previously described.[24] We used an Alexa488-conjugated mouse antihuman pERK1/2 antibody (Cell Signaling, Danvers, MA) at a final staining concentration of 0.05 μg/ml and the appropriate isotype control.

Flow Cytometric Measurement of CXCR2 Expression

Neutrophils were exposed to volatile anesthetics (see Preconditioning with Volatile Anesthetics). Following exposure for 45 min, they were stained on ice for 30 min with antibodies reactive to IL-8 receptor CXCR2 (mouse antihuman CXCR2 at a final staining concentration of 0.25 μg/ml; BD Biosciences). Appropriate isotype controls were run with every experiment. 7-AAD serving as a dead/live-stain was included in every sample, and only cells negative for 7-AAD were included in the analysis.

Neutrophil Migration Assay with Matrigel

Filters of a 96-well migration plate with 8 μm pores (Millipore, Billerica, MA) were coated with 10 μl of pure Matrigel (BD Biosciences). Matrigel is rich in collagen IV, laminin, and proteoglycans, and is widely used to simulate the extracellular matrix *in vitro* during invasion and migration.[25–27] Neutrophil were seeded in the upper compartment, preconditioned with volatile anesthetics, and stimulated with IL-8 (see Stimulation of Neutrophils). After stimulation for 15 min, 150 μl of Ham's F-12 medium supplemented with 10% fetal calf serum (Invitrogen) was added to the lower compartment and neutrophils were allowed to migrate for 2 h at 37°C. Transmigrated neutrophils were quantified by endogenous β-glucuronidase.[28] For each experiment, outliers that deviated more than one SD from the mean of each experimental group were excluded.

MC-38GFP (Green Fluorescent Protein)/Mouse Neutrophil Comigration Assay with Matrigel

Cell Culture of Mouse Neutrophils. *In vitro* differentiated mouse granulocytes were generated as described before.[29] Briefly, isolated bone marrow cells were transfected with murine stem cell virus vector containing estrogen dependent fusion oncoprotein (E2A-Pbx1-ER) that blocks myeloid differentiation. Conditionally immortalized myeloid progenitors were cultivated in the Roswell Park Memorial Institute medium (Invitrogen) supplemented with granulocyte macrophage colony-stimulating factor and 1 μM β-Estradiol. On removal of estrogen, progenitor clones differentiated to granulocytes and were characterized with staining of CD11b, Gr-1, and F4/80 antibodies.

Mouse colon carcinoma cell line MC-38GFP were grown in Dulbecco's Modified Eagle medium with 10% fetal calf serum.[30] Doubling time of MC-38GFP in culture is approximately 12 h.

Migration. Mouse neutrophils (2×10^5) were seeded on Matrigel-coated 24-well migration plates with 8 μm pores (BD Biosciences) and preconditioned with volatile anesthetics (as described for human granulocytes in Preconditioning with Volatile Anesthetics). Stimulation was performed with 100 ng/ml CXCL1 (recombinant mouse CXCL1, R&D Systems) for 15 min. Starved MC-38GFP cells (2×10^4) were then added to the granulocytes. Medium containing 10% fetal calf serum served as unspecific chemoattractant and was added to the lower compartment. Cells were allowed to migrate for 6 h at 37°C in an incubator free of volatile anesthetics.

Analysis. Nonmigrated cells were removed from the upper chamber with a cotton swab. Filters were fixed with paraformaldehyde and stained with diamidinophenylindole. Sixteen automatically randomized pictures of each sample were taken on a Leica LX microscope (Leica, Buffalo Grove, IL) and GFP/diamidinophenylindole cells counted. Data are given as total number of migrated cells.

Statistical Analysis

All experiments were performed at least three times with blood from different donors. For experiments with $n = 6$ or more for each condition, normal distribution was assessed using the Kolmogorov–Smirnov-test. Normally distributed values were expressed as mean ± SD and analyzed using one-way ANOVA with a Bonferroni *post hoc* correction to reduce the probability of a type I error. For zymography experiments with $n = 3$ for each condition, nonparametric testing with Kruskall-Wallis test was performed. Values are expressed as median and interquartiles.

$P < 0.05$ was considered significant. Statistical analyses were calculated with PASW 19 for OSX (SPSS Inc., Chicago, IL). Graphs were made with Graphpad Prism 5 for Macintosh (Graphpad Software, La Jolla, CA).

Results

Isolation of Neutrophils

The isolation procedure yielded neutrophils with a purity of more than 94% with less than 1% of dead cells (defined as 7-AAD+ cells) and less than 2.5% of apoptotic cells (defined as 7-AAD-/Annexin+ cells). No artificial activation of neutrophils occurred.

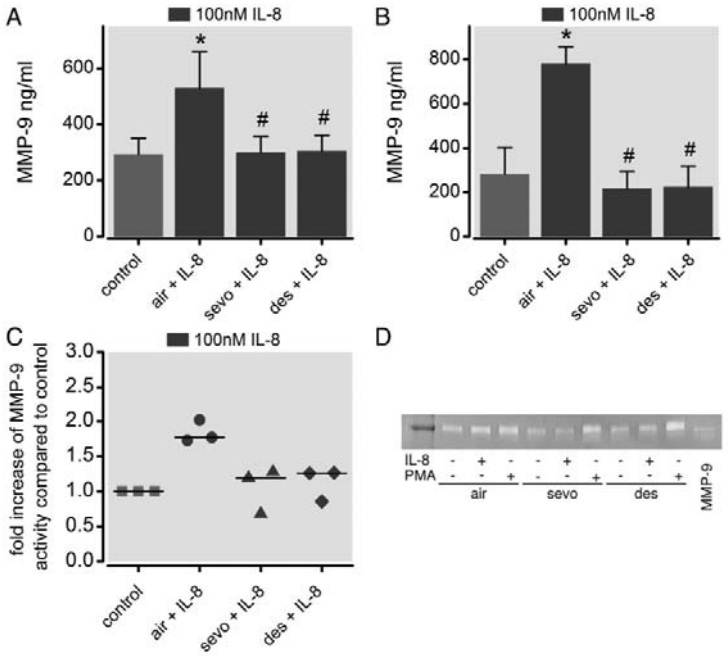


Fig. 1. (*A*) Matrix metalloproteinase-9 (MMP-9) release from neutrophils. Neutrophils were preconditioned with 2.2% sevoflurane (sevo + interleukin-8 [IL-8]), 6% desflurane (des + IL-8), or an air/5% CO_2 mixture (air + IL-8) for 45 min at 37°C. Neutrophils were then moved to an air/5% CO_2-flooded incubator free of volatile anesthetics and stimulated with 100 nM IL-8 for 15 min. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. Control refers to neutrophils that were exposed to air/5%CO_2 during preconditioning and stimulated for 15 min with phosphate-buffered saline instead of IL-8. *$P < 0.01$ for control *versus* air + IL-8, #$P < 0.01$ for air + IL-8 *versus* sevo + IL-8/des + IL-8. (*B*) Preconditioning with reduced volatile concentrations. Neutrophils were preconditioned with 1.1% sevoflurane (sevo + IL-8), 3% of desflurane (des + IL-8), or an air/5% CO_2 mixture (air + IL-8) followed by stimulation with IL-8 for 15 min. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. Control refers to neutrophils exposed to air/5% CO_2 during preconditioning followed by stimulation with phosphate-buffered saline instead of IL-8. *$P < 0.001$ for control *versus* air + IL-8, #$P < 0.001$ for air + IL-8 *versus* sevo + IL-8/des + IL-8. (*C*) Enzymatic activity of MMP-9. Enzymatic activity of MMP-9 in supernatants from neutrophils following preconditioning as described for Fig. 1A and stimulation with IL-8 was assessed by zymography. Data from three separate experiments (n = 3 for each variable) are presented. Control refers to neutrophils exposed to air/5% CO_2 and stimulated for 15 min with phosphate-buffered saline instead of IL-8. $P = 0.083$ for control *versus* air + IL-8/sevo + IL-8/des + IL-8. (*D*) Representative zymography gel. The MMP-9 band was identified by their molecular weight (dark band represents marker at 92 kDa) and human recombinant MMP-9 as loading control (outer right band, MMP-9). PMA = phorbol myristate acetate.

Volatile Anesthetics Reduce Release of Neutrophilic MMP-9

Stimulation of neutrophils under control conditions (air/5% CO_2) with 100 nM IL-8 for 15 min lead to a significant increase in MMP-9 (fig. 1A; control *vs.* air + IL-8, $P = 0.005$). When preconditioned with 2.2% of sevoflurane or 6% of desflurane for 45 min, release of MMP-9 upon stimulation was completely blunted. Compared with IL-8 stimulation with an air/5% CO_2-mixture incubation, preconditioning with volatile anesthetics decreased release of MMP-9 upon IL-8 stimulation by 41% (±13, 5%) and 40% (±13%), respectively (air + IL-8 *vs.* sevo + IL-8, $P = 0.007$, air + IL-8 *vs.* des + IL-8, $P < 0.001$). A comparable effect of preconditioning was also observed with reduced concentrations of volatile anesthetic of 1.1% sevoflurane or 3% desflurane (fig. 1B; air + IL-8 *vs.* sevo + IL-8/des+IL-8, $P < 0.001$).

Enzymatic Activity of MMP-9 Corresponds to MMP-9 Protein Expression

To quantify enzymatic activity of MMP-9, zymography was performed. Enzymatic activity was influenced by preconditioning with volatile anesthetics comparable with the effect on MMP-9 protein (fig. 1C, median and interquartile range; control 1.0 [0], air + IL-8 1.76 [0.29], sevo + IL-8 1.19

[0.6], des + IL-8 1.26 [0.4]; $P = 0.083$). A representative zymography gel is shown in figure 1D.

Changes in Release of MMP-9 Are Not Because of Cytotoxic Effect of Volatile Anesthetics

Neutrophil viability was assessed to exclude a cytotoxic effect of preconditioning with volatile anesthetics. No differences in 7-AAD negative cells were noted with viability rates more than 92% in all groups (data not shown).

PKC-dependent Phosphorylation of ERK1/2 and Release of MMP-9 Is Not Altered by Volatile Anesthetics

Direct stimulation of PKC with PMA induced a strong phosphorylation of ERK1/2 in all groups (fig. 2A; control *vs.* air + PMA/sevo + PMA/des + PMA, $P < 0.001$). Preconditioning with volatile anesthetics did not influence ERK1/2-phosphorylation through PKC (air + PMA *vs.* sevo + PMA, −18.6% [±26.5%], $P = 0.37$; air + PMA *vs.* des + PMA, −19.5% [±17.8%]; $P = 0.27$). No difference was noted between sevoflurane and desflurane preconditioning (sevo + PMA *vs.* des + PMA, $P = 1.0$).

As a consequence, preconditioning did not alter release of MMP-9 from PMA-stimulated neutrophils (fig. 2B, air + PMA *vs.* sevo + PMA, +5.2% [±12.2%]; air + PMA *vs.* des + PMA, +8.8% [±25.6%], $P = 1.0$).

Protection with Volatile Anesthetics Is Also Observed Upon Activation of IL-8 Receptor CXCR2

Stimulation with 100 ng/ml of CXCL1 induced a 51% (±26.2%) increase in MMP-9 release (fig. 3, control *vs.* air + CXCL1, $P = 0.002$). Preconditioning with volatile anesthetics for 45 min before stimulation with CXCL1 significantly reduced MMP-9 release by 29% (±14.8%) and 42% (±10.7%), respectively (air + CXCL1 *vs.* sevo + CXCL1, $P = 0.009$; air + CXCL1 *vs.* des + CXCL1, $P < 0.001$).

Volatile Anesthetics Do Not Alter Expression of the IL-8 Receptor CXCR2

To further assess the role of IL-8 receptor CXCR2 in mediating volatile anesthetic preconditioning, surface expression of CXCR2 was quantified following exposure to volatile anesthetics. Neither sevoflurane nor desflurane altered CXCR2 expression (fig. 4).

Preconditioning Reduces Matrigel Permeability Through MMP-9 Down-regulation

In the current study, higher MMP-9 levels following IL-8 stimulation lead to an increase in permeability of the matrigel layer for neutrophils (fig. 5; control *vs.* air + IL-8, +31.5% [±36.5%], $P < 0.001$). Preconditioning prevented this increase and reduced neutrophil migration to unstimulated levels (air + IL-8 *vs.* sevo + IL-8, $P = 0.011$; air + IL-8 *vs.* des + IL-8, $P < 0.001$).

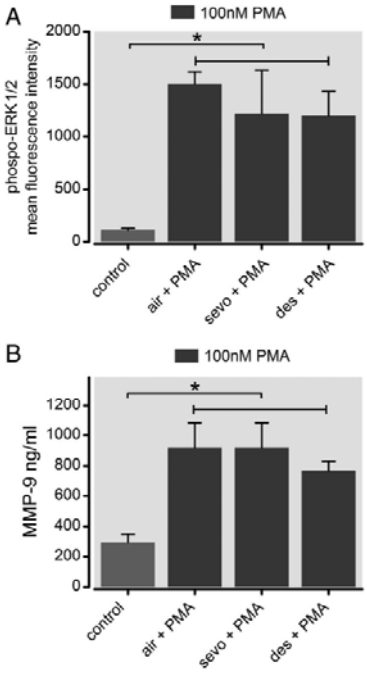


Fig. 2. (*A*) Protein kinase C-dependent phosphorylation of extracellular regulated kinase (ERK)1/2. Neutrophils were preconditioned with volatile anesthetics for 45 min and then stimulated with 100 nM phorbol myristate acetate (PMA) for 5 min in an air/5% CO_2-flooded incubator. Phosphorylation of ERK 1/2 was quantified by flow cytometry. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. *$P < 0.001$ for control *versus* air + PMA/sevo + PMA/des + PMA. (*B*) Protein kinase C-dependent matrix metalloproteinase-9 release. Following preconditioning, neutrophils were stimulated with PMA for 5 min in an air/5% CO_2-flooded incubator. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. *$P < 0.001$ for control *versus* air + PMA/sevo + PMA/des + PMA. Des = desflurane; MMP = matrix metalloproteinase-9; phospho-ERK 1/2 = dependent phosphorylation of extracellular regulated kinase 1/2; PMA = phorbol myristate acetate; Sevo = sevoflurane.

Preconditioning of Neutrophils Reduces Tumor Cell Transmigration

Transmigration through extracellular matrix is required during metastatic seeding. As preconditioning reduced the permeability of Matrigel for neutrophils, the impact on tumor cell transmigration was evaluated. MC-38GFP cells, derived from mouse colon carcinoma, were allowed to migrate in the presence of neutrophils either with or without preconditioning. Stimulation of neutrophils strongly increased tumor cell transmigration (fig. 6; control *vs.* air + CXCL1, $P < 0.001$).

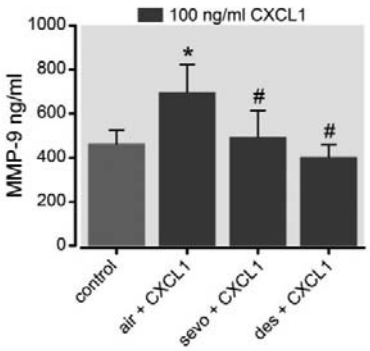


Fig. 3. Stimulation of the interleukin-8 CXC-receptor-2 with CXC-ligand 1 (CXCL1). Neutrophils were preconditioned with 2.2% sevoflurane (sevo + CXCL1), 6% desflurane (des + CXCL1), or an air/5% CO_2 mixture (air + CXCL1), followed by stimulation with 100 nM CXCL1 in an air/5% CO_2-flooded incubator. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. *P < 0.01 for control *versus* air + CXCL1, #P < 0.01 for air + CXCL1 *versus* sevo + CXCL1/des + CXCL1. CXCL1 = CXC-ligand 1; MMP = matrix metalloproteinase.

Preconditioning of neutrophils reduced the number of transmigrated tumor cells through Matrigel to baseline levels (air + CXCL1 *vs.* sevo + CXCL1/des + CXCL1, P < 0.001).

Discussion

Metastatic disease is one of the main factors affecting survival in patients with cancer.[31] MMP-9 was demonstrated to be correlated with tumor invasion and metastatic seeding.[10,11,32] This study provides evidence that preconditioning with sevoflurane or desflurane at clinically used concentrations reduces the release of MMP-9 from human neutrophils in an *in vitro* model of reperfusion injury. The effect by which volatile anesthetics reduce the release of MMP-9 is located downstream of the IL-8 receptor CXCR2, but upstream of PKC. Through down-regulation of MMP-9, preconditioning also reduces the migration of neutrophils and the invasiveness of tumor cells across the extracellular matrix.

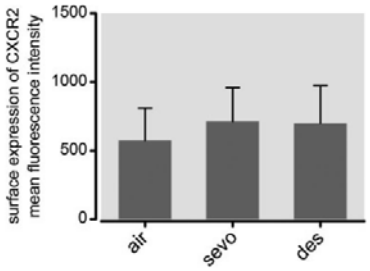


Fig. 4. Interleukin-8 CXC-receptor-2 (CXCR2). Neutrophils were preconditioned with volatile anesthetics for 45 min, followed by staining on ice with anti-CXCR2 antibodies for 30 min. Expression of CXCR2 was analyzed by flow cytometry. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. Des = desflurane; Sevo = sevoflurane.

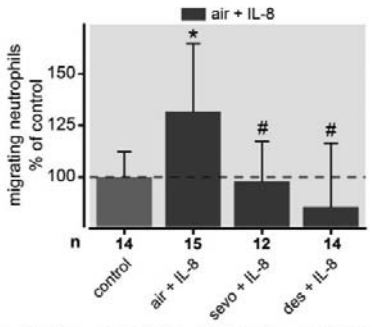


Fig. 5. Matrigel permeability. Neutrophils were preconditioned and stimulated on Matrigel-coated migration filters. Neutrophils were allowed to migrate for 2 h in an incubator free of volatile anesthetics. Data from four separate experiments, each condition repeated in quadruples, are given. Outliers, defined as deviating more than 1 SD from the mean of the group for each separate experiment, were excluded from further analysis. Final number of samples in each variable is given in the figure. Bars show mean and SD. *P < 0.001 for control *versus* air + interleukin-8 (IL-8), #P < 0.05 for air + IL-8 *versus* sevo + IL-8/des + IL-8. Des = desflurane; IL-8 = interleukin-8; Sevo = sevoflurane.

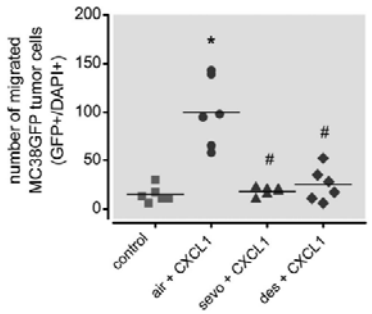


Fig. 6. Invasion of MC-38 green fluorescent protein tumor cells. Mouse neutrophils were preconditioned and stimulated with CXC-ligand 1 (CXCL1). MC-38 green fluorescent protein tumor cells were added and allowed to migrate for 6 h. Scatter plot shows median from six separate migration filters for each condition (n = 6 for each condition). *P <0.001 for control *versus* air + CXCL1, #P < 0.001 for air + CXCL1 *versus* sevo + CXCL1/des + CXCL1. CXCL1 = CXC-ligand 1; DAPI = diamidinophenylindole; Des = desflurane; MC38GFP = MC-38 green fluorescent protein tumor cells; Sevo = sevoflurane.

The perioperative period is of great interest regarding the risk for recurrence after surgery for colorectal cancer. Only recently, circulating tumor cells measured postoperatively were identified as independent predictors of recurrence of colorectal cancer.[33] To form metastasis, these tumor cells need to extravasate from the bloodstream and migrate through extracellular matrix and basement membrane. MMP-9 promotes this process, first through direct degradation of extracellular matrix and then through the formation of premetastatic niches.[34] Reducing MMP-9-release through preconditioning with volatile anesthetics might therefore come just in time before circulating tumor cells seed to the target organ.

This work demonstrates that preconditioning with volatile anesthetics reduces the release of MMP-9 from IL-8-stimulated neutrophils. The currently commercially available ELISA kits do not distinguish between the inactive pro-MMP-9 and its active form, but rather measure total MMP-9. MMP-9 concentrations in the current study correlated to MMP-9 activity as evidenced by zymography, further validating the ELISA results.

Volatile anesthetics were often demonstrated to attenuate neutrophil activation *in vitro*[19,21] and *in vivo*.[17,18,35] The observed attenuation of MMP-9 is unique since in contrast to neutrophil surface molecules involved in activation and adhesion such as integrins and selectins, MMP-9 is regulated at the level of tertiary granule release, a subgroup of the peroxidase-negative granules.[36,37] IL-8 stimulates the release of MMP-9 through activation of PKC, which in turn phosphorylates ERK1/2.[12] The direct stimulation of PKC with PMA led to a strong phosphorylation of ERK1/2 in our setting. However, preconditioning did not affect this process. Similarly, MMP-9 release from PMA-stimulated neutrophils was not altered by preconditioning with volatile anesthetics. We therefore conclude that volatile anesthetic preconditioning does not directly interfere with tertiary granule release, but rather affects stimulatory pathways upstream of PKC.

Most neutrophil responses to IL-8, such as cell adhesion or release of primary granules, are triggered through activation of both IL-8 receptors CXCR1 and CXCR2.

In contrast, tertiary granule release was shown to rely exclusively on activation of CXCR2.[12] CXCL1 and IL-8 both belong to the family of CXC-cytokines. However, CXCL1 binds to CXCR2 only.[38] Accordingly, we found that stimulation of CXCR2 with CXCL1 led to an increase in MMP-9. Similarly to the combined activation of CXCR1 and CXCR2 with IL-8, MMP-9 release from CXCL1-stimulated neutrophils was inhibited by the application of volatile anesthetics. This suggests that CXCR1 is not necessary to mediate the effect of preconditioning with volatile anesthetics on tertiary granule release.

Only surface-bound CXCR2 is able to form ligand-receptor complexes with IL-8 or CXCL1, resulting in internalization of the complex and downstream signaling. But internalization is also thought to act as a stop mechanism to reduce neutrophil activity once the neutrophil reached the site of inflammation.[39] No differences in CXCR2 expression following volatile anesthetic preconditioning were found in the current study. This argues against a direct interaction between the volatile anesthetic and CXCR2 that might have triggered internalization of the receptor.

Downstream events following stimulation of CXCR2 with IL-8 include the rapid increase in intracellular calcium.[40] Intracellular calcium triggers a wide range of neutrophil responses in inflammation that are influenced by volatile anesthetics, such as oxidative burst[20,41] and integrin up-regulation and adhesion.[19,42] In contrast to those, the release of MMP-9 was demonstrated to be independent from changes in intracellular calcium concentration.[12] This suggests that preconditioning with volatile anesthetics regarding tertiary granule release is mediated through calcium-independent pathways.

Finally, the biologic relevance of the observed differences in MMP-9 release on the integrity of the extracellular matrix was investigated. Matrigel is derived from Engelbreth-Holm-Swarm sarcoma and consists mostly of collagen IV, laminin, and proteoglycans.[26] It is widely used as an equivalent for extracellular matrix when studying tumor invasion.[25,27] MMP-9 degrades components of Matrigel, and the migration of invasive cells through Matrigel has previously been shown to rely on MMP-9 expression.[43,44] The current study demonstrated that down-regulation of MMP-9 by preconditioning with volatile anesthetics reduced the number of neutrophils that were able to cross the extracellular matrix.

It seems reasonable that invasive tumor cells as well should be influenced by different extents of extracellular matrix degradation. This is supported by Sun *et al.*, who demonstrated that the migration of oral squamous cell carcinoma cells through Matrigel is almost completely dependent on the tumors' ability to secrete MMP-9, and failing to do so suppresses the transmigration process.[45] Therefore, this investigation focused on the MC38 cell line that is derived from mouse colon cancer, as metastatic seeding for these tumor cells was demonstrated to be dependent on MMP-9 expression.[7] Neutrophil preconditioning hindered the subsequent migration process and reduced the number of tumor cells that successfully crossed the extracellular matrix barrier. This experimental setup clearly underlines the functional consequence of decreased neutrophilic MMP-9 secretion upon exposure to volatile anesthetics *in vitro*.

The use of Matrigel is currently the most common model for studying tumor invasion *in vitro*. This is mostly because of the lack of tissue preparation heterogeneity that is observed with other substrates such as amnion or chorioallantoic membranes, resulting in difficult reproducibility of these models. Although routinely used, there might be additional, unknown components of extracellular matrix that are lacking in the Matrigel preparation. Therefore, further *in vivo* studies

are clearly warranted before making clinical conclusions of Matrigel-derived invasion studies.

In vitro studies on volatile anesthetics face two problems: first, standard concentrations for volatile anesthetics are usually defined according to the patient's response to the anesthetic, and might not be simply transferable to cell experiments. In the current study it was decided to adhere to the clinically most relevant concentrations of 0.5 or 1 minimum alveolar concentration, as used by many other authors as well.[19,20] Second, the experimental setup of exposure to volatile anesthetics might influence the absolute concentration of anesthetic that can reach the cells. However, the current method of cell exposure to volatile anesthetics is a well-established procedure in our lab that has yielded well reproducible results.[46–49]

In conclusion, this study demonstrates that volatile anesthetics attenuate IL-8-induced MMP-9 release from tertiary granules downstream of CXCR2 and reduce the migratory behavior of neutrophils in Matrigel. Consequently, the invasive behavior of tumor cells facilitated through neutrophils has been abrogated. Although these first *in vitro* results are promising, further studies are warranted to evaluate the role of volatile anesthetics in tumor cell migration and spreading *in vivo*.

The authors thank Christa Booy and Livia Reyes, Technicians, University of Zurich, Institute of Physiology, Zurich, Switzerland, for technical support, and Sereina Graber, Master's Candidate, Institute of Biology, University of Zurich, Zurich, Switzerland, for statistical support.

References

1. Chari R: Colorectal Cancer Metastatic to the Liver: Resection and Intraarterial Chemotherapy. In: Cameron JL, ed. Current Surgical Therapy, 7th ed. St. Louis: Mosby; 2001, pp 375–80
2. Guller U, Zajac P, Schnider A, Bösch B, Vorburger S, Zuber M, Spagnoli GC, Oertli D, Maurer R, Metzger U, Harder F, Heberer M, Marti WR: Disseminated single tumor cells as detected by real-time quantitative polymerase chain reaction represent a prognostic factor in patients undergoing surgery for colorectal cancer. Ann Surg 2002; 236:768–75
3. Yamaguchi K, Takagi Y, Aoki S, Futamura M, Saji S: Significant detection of circulating cancer cells in the blood by reverse transcriptase-polymerase chain reaction during colorectal cancer resection. Ann Surg 2000; 232:58–65
4. Doi K, Horiuchi T, Uchinami M, Tabo T, Kimura N, Yokomachi J, Yoshida M, Tanaka K: Hepatic ischemia-reperfusion promotes liver metastasis of colon cancer. J Surg Res 2002; 105:243–7
5. Ku Y, Kusunoki N, Shiotani M, Maeda I, Iwasaki T, Tominaga M, Kitagawa T, Fukumoto T, Suzuki Y, Kuroda Y: Stimulation of haematogenous liver metastases by ischaemia-reperfusion in rats. Eur J Surg 1999; 165:801–7
6. Yoshida M, Horiuchi T, Uchinami M, Tabo T, Kimura N, Yokomachi J, Doi K, Nakamura T, Tamagawa K, Tanaka K: Intermittent hepatic ischemia-reperfusion minimizes liver metastasis in rats. J Surg Res 2003; 111:255–60
7. Nicoud IB, Jones CM, Pierce JM, Earl TM, Matrisian LM, Chari RS, Gorden DL: Warm hepatic ischemia-reperfusion promotes growth of colorectal carcinoma micrometastases in mouse liver *via* matrix metalloproteinase-9 induction. Cancer Res 2007; 67:2720–8
8. Nagase H, Woessner JF Jr: Matrix metalloproteinases. J Biol Chem 1999; 274:21491–4
9. Fidler IJ: Molecular biology of cancer: Invasion and metastasis. In: DeVita VT, ed. Cancer: Principles and Practise of Oncology, 5th ed. Philadelphia: Lippincott-Raven; 1997, pp 135–52
10. Bendardaf R, Buhmeida A, Hilska M, Laato M, Syrjänen S, Syrjänen K, Collan Y, Pyrhönen S: MMP-9 (gelatinase B) expression is associated with disease-free survival and disease-specific survival in colorectal cancer patients. Cancer Invest 2010; 28:38–43
11. Buhmeida A, Bendardaf R, Hilska M, Collan Y, Laato M, Syrjänen S, Syrjänen K, Pyrhönen S: Prognostic significance of matrix metalloproteinase-9 (MMP-9) in stage II colorectal carcinoma. J Gastrointest Cancer 2009; 40:91–7
12. Chakrabarti S, Patel KD: Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils. J Leukoc Biol 2005; 78:279–88
13. Inci I, Erne B, Arni S, Jungraithmayr W, Inci D, Hillinger S, Vogt P, Leskosek B, Weder W: Prevention of primary graft dysfunction in lung transplantation by N-acetylcysteine after prolonged cold ischemia. J Heart Lung Transplant 2010; 29:1293–301
14. Kukielka GL, Smith CW, LaRosa GJ, Manning AM, Mendoza LH, Daly TJ, Hughes BJ, Youker KA, Hawkins HK, Michael LH: Interleukin-8 gene induction in the myocardium after ischemia and reperfusion *in vivo*. J Clin Invest 1995; 95:89–103
15. Sekido N, Mukaida N, Harada A, Nakanishi I, Watanabe Y, Matsushima K: Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8. Nature 1993; 365:654–7
16. Beck-Schimmer B, Breitenstein S, Urech S, De Conno E, Wittlinger M, Puhan M, Jochum W, Spahn DR, Graf R, Clavien PA: A randomized controlled trial on pharmacological preconditioning in liver surgery using a volatile anesthetic. Ann Surg 2008; 248:909–18
17. Heindl B, Reichle FM, Zahler S, Conzen PF, Becker BF: Sevoflurane and isoflurane protect the reperfused guinea pig heart by reducing postischemic adhesion of polymorphonuclear neutrophils. ANESTHESIOLOGY 1999; 91:521–30
18. Nader ND, Li CM, Khadra WZ, Reedy R, Panos AL: Anesthetic myocardial protection with sevoflurane. J Cardiothorac Vasc Anesth 2004; 18:269–74
19. de Rossi LW, Horn NA, Buhre W, Gass F, Hutschenreuter G, Rossaint R: The effect of isoflurane on neutrophil selectin and beta(2)-integrin activation *in vitro*. Anesth Analg 2002; 95:583–7
20. Fröhlich D, Rothe G, Schwall B, Schmid P, Schmitz G, Taeger K, Hobbhahn J: Effects of volatile anaesthetics on human neutrophil oxidative response to the bacterial peptide FMLP. Br J Anaesth 1997; 78:718–23
21. Möbert J, Zahler S, Becker BF, Conzen PF: Inhibition of neutrophil activation by volatile anesthetics decreases adhesion to cultured human endothelial cells. ANESTHESIOLOGY 1999; 90:1372–81
22. Roos D, de Boer M: Purification and cryopreservation of phagocytes from human blood. Methods Enzymol 1986; 132:225–43
23. Hu X, Beeton C: Detection of functional matrix metalloproteinases by zymography. J Vis Exp 2010; 45:2445
24. Trampont P, Zhang L, Ravichandran KS: ShcA mediates the dominant pathway to extracellular signal-regulated kinase activation during early thymic development. Mol Cell Biol 2006; 26:9035–44
25. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN: A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res 1987; 47:3239–45

26. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR: Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 1982; 21:6188–93
27. Terranova VP, Hujanen ES, Loeb DM, Martin GR, Thornburg L, Glushko V: Use of a reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells. Proc Natl Acad Sci U S A 1986; 83:465–9
28. Ludwig A, Petersen F, Zahn S, Götze O, Schröder JM, Flad HD, Brandt E: The CXC-chemokine neutrophil-activating peptide-2 induces two distinct optima of neutrophil chemotaxis by differential interaction with interleukin-8 receptors CXCR-1 and CXCR-2. Blood 1997; 90:4588–97
29. Sykes DB, Kamps MP: Estrogen-dependent E2a/Pbx1 myeloid cell lines exhibit conditional differentiation that can be arrested by other leukemic oncoproteins. Blood 2001; 98: 2308–18
30. Borsig L, Wong R, Hynes RO, Varki NM, Varki A: Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc Natl Acad Sci U S A 2002; 99: 2193–8
31. Hart I: The spread of tumours. In: Knowles MA, ed. Introduction to the Cellular and Molecular Biology of Cancer, 4th ed. Oxford: Oxford University Press; 2007, pp 278–87
32. Liu Z, Li L, Yang Z, Luo W, Li X, Yang H, Yao K, Wu B, Fang W: Increased expression of MMP9 is correlated with poor prognosis of nasopharyngeal carcinoma. BMC Cancer 2010; 10:270
33. Peach G, Kim C, Zacharakis E, Purkayastha S, Ziprin P: Prognostic significance of circulating tumour cells following surgical resection of colorectal cancers: A systematic review. Br J Cancer 2010; 102:1327–34
34. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D: VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 2005; 438:820–7
35. Lucchinetti E, Ambrosio S, Aguirre J, Herrmann P, Härter L, Keel M, Meier T, Zaugg M: Sevoflurane inhalation at sedative concentrations provides endothelial protection against ischemia-reperfusion injury in humans. ANESTHESIOLOGY 2007; 106:262–8
36. Opdenakker G, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, Proost P, Van Damme J: Gelatinase B functions as regulator and effector in leukocyte biology. J Leukoc Biol 2001; 69:851–9
37. Borregaard N, Cowland JB: Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997; 89: 3503–21
38. Ahuja SK, Murphy PM: The CXC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. J Biol Chem 1996; 271:20545–50
39. Rose JJ, Foley JF, Murphy PM, Venkatesan S: On the mechanism and significance of ligand-induced internalization of human neutrophil chemokine receptors CXCR1 and CXCR2. J Biol Chem 2004; 279:24372–86
40. Jones SA, Dewald B, Clark-Lewis I, Baggiolini M: Chemokine antagonists that discriminate between interleukin-8 receptors. Selective blockers of CXCR2. J Biol Chem 1997; 272: 16166–9
41. Nakagawara M, Takeshige K, Takamatsu J, Takahashi S, Yoshitake J, Minakami S: Inhibition of superoxide production and Ca2+ mobilization in human neutrophils by halothane, enflurane, and isoflurane. ANESTHESIOLOGY 1986; 64:4–12
42. Lawson MA, Maxfield FR: Ca(2+)- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. Nature 1995; 377:75–9
43. Gong Y, Hart E, Shchurin A, Hoover-Plow J: Inflammatory macrophage migration requires MMP-9 activation by plasminogen in mice. J Clin Invest 2008; 118:3012–24
44. Sun HC, Qiu ZJ, Liu J, Sun J, Jiang T, Huang KJ, Yao M, Huang C: Expression of hypoxia-inducible factor-1 alpha and associated proteins in pancreatic ductal adenocarcinoma and their impact on prognosis. Int J Oncol 2007; 30:1359–67
45. Sun L, Diamond ME, Ottaviano AJ, Joseph MJ, Ananthanarayan V, Munshi HG: Transforming growth factor-beta 1 promotes matrix metalloproteinase-9-mediated oral cancer invasion through snail expression. Mol Cancer Res 2008; 6:10–20
46. Yue T, Roth Z'graggen B, Blumenthal S, Neff SB, Reyes L, Booy C, Steurer M, Spahn DR, Neff TA, Schmid ER, Beck-Schimmer B: Postconditioning with a volatile anaesthetic in alveolar epithelial cells *in vitro*. Eur Respir J 2008; 31:118–25
47. Urner M, Limbach LK, Herrmann IK, Müller-Edenborn B, Roth-Z'graggen B, Schlicker A, Reyes L, Booy C, Hasler M, Stark WJ, Beck-Schimmer B: Fluorinated groups mediate the immunomodulatory effects of volatile anesthetics in acute cell injury. Am J Respir Cell Mol Biol 2011; 45:617–24
48. Steurer M, Schläpfer M, Z'graggen BR, Booy C, Reyes L, Spahn DR, Beck-Schimmer B: The volatile anaesthetic sevoflurane attenuates lipopolysaccharide-induced injury in alveolar macrophages. Clin Exp Immunol 2009; 155:224–30
49. Suter D, Spahn DR, Blumenthal S, Reyes L, Booy C, Z'graggen BR, Beck-Schimmer B: The immunomodulatory effect of sevoflurane in endotoxin-injured alveolar epithelial cells. Anesth Analg 2007; 104:638–45